IN VITRO AND *IN VIVO* EVALUATION OF NOVEL I-DOMAIN CONJUGATES FOR DRUG TARGETING TO IMMUNE CELLS AND SUPPRESSION OF EAE IN MICE

By

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Submitted to the Department of Pharmaceutical Chemistry and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dissertation Defended on June 7, 2010

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In vitro and _in vivo_ evaluation of novel I-domain conjugates for drug targeting to immune cells and suppression of EAE in mice

Prakash Manikwar

The University of Kansas, 2010

The long-term objective of this project is to utilize the I-domain of leukocyte function associated antigen-1 (LFA-1) to target antigenic peptides and drugs to intercellular adhesion molecule-1 (ICAM-1) expressed on the surface of immune cells. The objectives of the dissertation are: 1) to characterize the binding properties of the I-domain to ICAM-1 receptors on the surface of lymphocytes (Raji cells), and 2) to evaluate the efficacy of PLP-I-domain conjugates in suppressing experimental autoimmune encephalomyelitis (EAE) in the mouse model, an animal model for multiple sclerosis. To accomplish these objectives, the I-domain protein was labeled with fluorescein isothiocyanate (FITC) at several lysine residues to produce the FITC-I-domain. Along with trypsin digestion and peptide mapping, we utilized a specific fragmentation of the fluorochrome moiety from the modified residues in the electrospray ionization-mass spectrometry (ESI-MS) to identify the conjugation sites more quickly (Chapter 2). The FITC-I-domain binds to ICAM-1 in a calcium-, time- and energy-dependent manner. It enters the Raji cells via receptor-mediated endocytosis. FITC-I-domain binding to ICAM-1 was blocked by anti-I-domain mAb; in contrast, anti-ICAM-1 mAb to D1 and D2-domain enhance FITC-I-domain binding to ICAM-1 on the cell surface (Chapter 3). The I-domain protein was

conjugated to an antigenic peptide, $PLP_{139-151}$, to produce PLP-I-domain-1 and -2 conjugates. We evaluated the biological activities of the conjugates in female SJL/J mice induced with EAE (Chapter 4). The *in vivo* studies showed that PLP-I-domain-1 has excellent efficacy in suppressing EAE, similar to that of the best positive control (i.e., Ac-PLP-cIBR1-NH_2). Although PLP-I-domain-2 could delay the onset of EAE compared to PBS, it was not as potent as PLP-I-domain-1. The chemical structure differences between PLP-I-domain-1 and -2 were determined using tryptic digestion followed by mass spectroscopic analysis. The number of conjugation sites in PLP-I-domain-1 is higher than in PLP-I-domain-2; this suggests that these additional sites in PLP-I-domain-1 contribute to its biological activity. In conclusion, the I-domain protein binds to and is internalized by ICAM-1 receptors on the surface of immune cells. The proper conjugation of PLP peptides to I-domain (i.e., PLP-I-domain-1) is necessary for suppressing EAE in the animal model.

Dedicated to:

My parents, Prabhakar & Shashekala Manikwar

My brother, Kiran Kumar Manikwar

My sister, Deepa Bukka

My friend, Pramod Kumar Allani

and

My wife, Pavithra Vani Nama

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TABLE OF CONTENTS

CHAPTER 1

Antigen-Specific Blocking of Immunological Synapse Formation using Bifunctional Peptide Inhibitors (BPI)

1.1 Immunological Synapse (IS)

1.1.1 Mechanism of formation of IS

One of the major events in triggering adaptive immunity is the activation of T cells. T cells are activated by the interaction of the T cell receptor (TCR) with the antigen that has been processed and presented by an APC via major histocompatibility gene complex (MHC).[1] Monks *et al.* (1998) were the first to report the formation of a three-dimensional cell-cell contact between a fixed single T cell and an antigen-presenting cell (APC).[2] This cell-cell contact is an interaction of surface receptors and intracellular proteins in a well-organized and spatially distributed manner, leading to the formation of two concentric rings termed "supramolecular activation clusters" (SMAC). The inner ring is referred as the central TCR-SMAC (c-SMAC or Signal-1). It is composed of protein kinase C (PKC-θ) surrounded by an outer or peripheral SMAC (p-SMAC or Signal-2) enriched mainly with leukocyte function-associated antigen-1 (LFA-1) and talin. Initial contact between the T cell and APC involving TCR and MHC-peptide (MHC-p) and other co-stimulatory molecules is called the "immunological synapse" (IS).[3] Grakoui *et al.* identified multiple stages of TCR engagement during the formation of stable IS in a dynamic environment. Initially, it involves central junction formation by adhesion molecules (*i.e.*, ICAM-1/LFA-1 or Signal-2) and peripheral TCR-MHC-p complex (Signal-1) in a nascent IS; this is followed by actin-based MHC-p transportation (translocation) to the center (Fig. 1). Finally, stabilization of the cluster takes place.

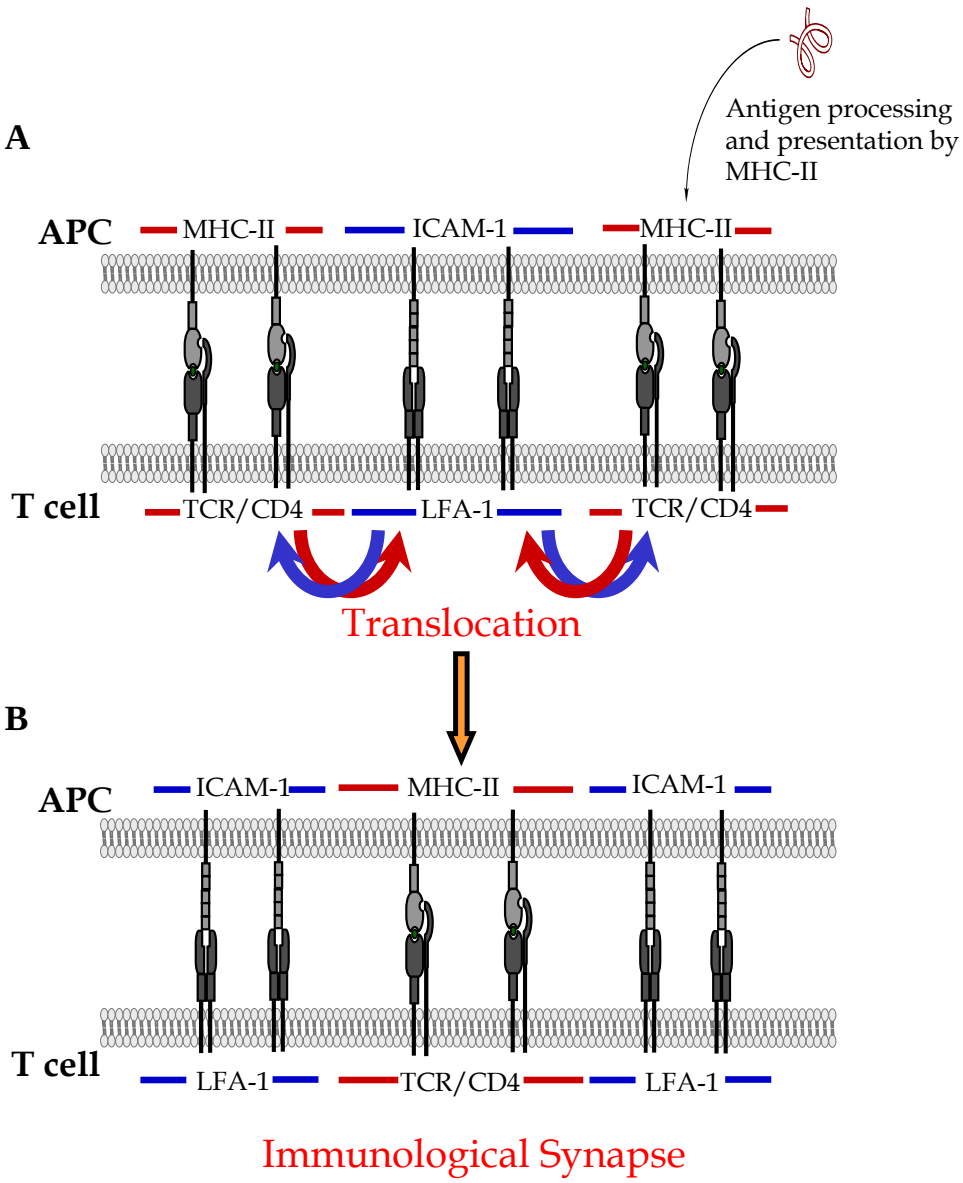

Figure 1. Mechanism of immunological synapse formation during T cell and APC interaction. **(A)** Initial contact between Signal-1 (TCR/MHC-II-peptide complex) and Signal-2 (LFA-1/ICAM-1 complex). **(B)** Translocation of Signal-1 and Signal-2 to form c-SMAC and p-SMAC of the immunological synapse.

Since its discovery, many molecules involved in IS have been reported, and several of these are ligand/receptor pairs. They include TCR (CD4 or CD8)-MHC-p complex; adhesion molecules (*i.e.*, LFA-1/intercellular adhesion molecule-1 (ICAM-1) and/or ICAM-2; ICAM-3/LFA-1 or DC-SIGN; CD2/LFA-3); positive and negative co-stimulatory molecules (CD28/B7-1 (CD80) and B7-2 (CD86); cytotoxic T lymphocyte antigen (CTLA-4)/B7-1 and B7-2; inducible co-stimulatory molecules (ICOS)/B7h; OX40/OX40L; RANKL/RANK; CD40L/CD40; 4-1BB/4-1BBL; programmed death (PD-1)/PD-L1/2; and CD27/CD70) molecules (Fig. 2).[4-6] The cytoskeletal protein talin and CD2-associated protein as well as intracellular signaling proteins such as PKC-θ, LcK, ZAP, Fyn and MEKK2 have also been identified.[6-7] The difference in their signaling processes has been attributed to the type of interacting co-stimulatory molecules; for example, the interaction could be an activation or inhibition, which affects the balance between effector and regulatory functions of a T cell.

There is a need to further understand the structure and function of the IS because of its dynamic nature of formation, which is an active process rather than just an accumulation of proteins involved in TCR signaling. Initially, it was thought that the IS is required for initiating, stabilizing, and sustaining TCR signaling. However, TCR-mediated tyrosine kinase signaling in T cells occurs even before the maturation of the IS, suggesting that T-cell activation takes place even before sustained signaling.[8-9] The formation of the IS was suggested to initiate multiple tasks, including organizing the secretion of cytokines and cytotoxic agents by $CD4^+$ and $CD8^+$ T cells, respectively, during their encounter with an APC.[9-14] The IS enhances the interaction of CD28/B7-1/2

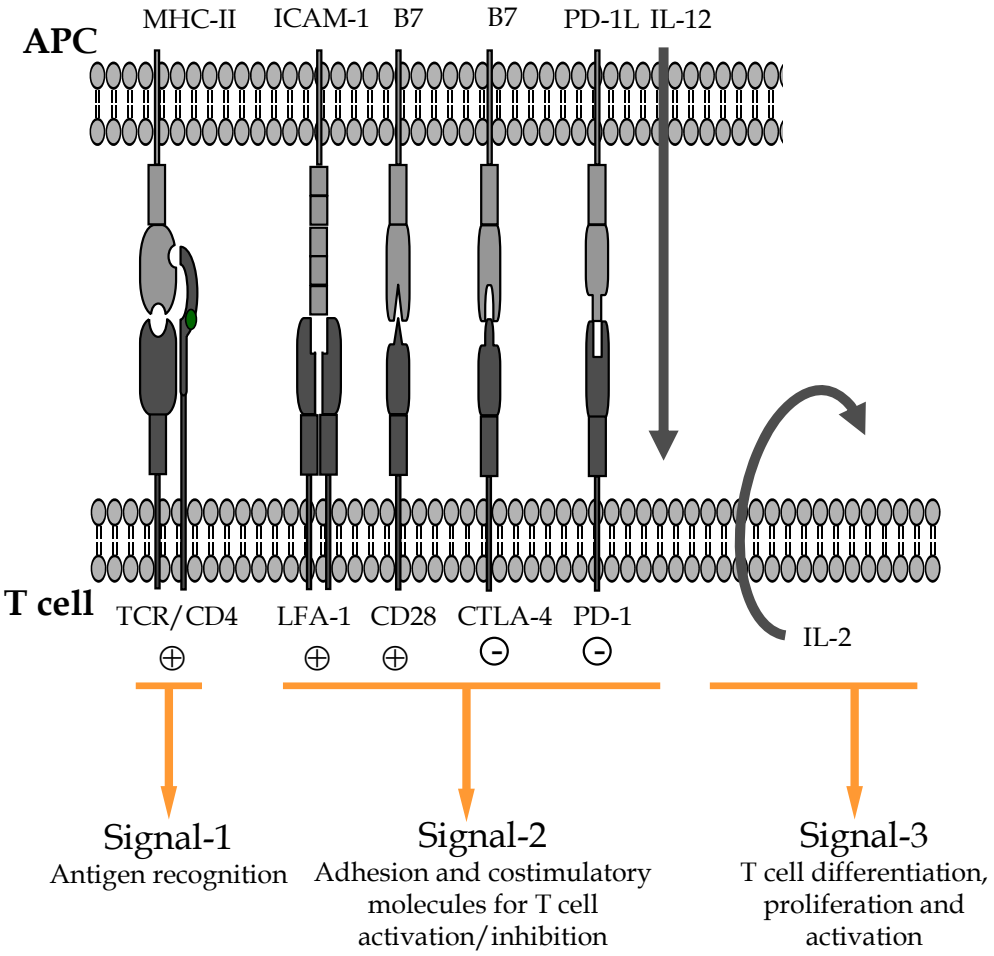

Figure 2. Signaling molecules involved in the interface of T cell and APC interaction. The interaction between T cell and APC involves several pairs of receptors (Signal-1 and -2) and is associated with the release of cytokines (Signal-3).

at the center.[9] Lezzi *et al*.[15] demonstrated that the activation and deletion of either naïve or effector T cells is dependent on the duration of antigenic stimulation. Prolonged antigenic stimulation is required for activation of naïve T cells, but it causes apoptosis in effector T cells. Furthermore, TCR clustering enhances binding with MHC-p, and the cSMAC is an important site for strong TCR signaling.[16,17] The internalization of TCR is required for down-regulation of TCR signaling.[16] The formation of IS may be required for gene activation to induce and secrete effector molecules and for intercellular transfer of MHC-p complex along with other receptors such as B7-1 from APC to T cell.[6, 18-19] This event may have biological repercussions, allowing T cells to behave as APC, altering the cellular functions and over all controlling the immune response itself.[20]

1.1.2 Signal-1

As described earlier, the formation of IS requires both initial antigen-specific (Signal-1) and distinct co-stimulatory signals (Signal-2) in the two-signal model (Fig. 2).[21-22] Earlier studies demonstrated that a complex formed by an immunogenic peptide and purified MHC-II (Ia) molecule was recognized by the TCR.[23-24] These findings were further confirmed by solving various crystal structures of peptide-MHC complexes.[25-33] Later, the specific TCR recognition of MHC-p complex on the surface of APC as a result of antigen processing was termed Signal-1.[21-22]

TCR is a disulfide-linked heterodimer belonging to the Ig supergene family in the CD3 superfamily, and it consists of α/β or γ/δ chains. Each chain is composed of two extracellular (one variable and one constant), a transmembrane, and a short cytoplasmic

domain.[34] The variable domain has the greatest diversity in the complementarity-determining region 3 (CDR3).[35] The sequence of CDR3 is primarily involved in recognizing the antigenic peptide presented by MHC molecules on APC.[36] MHC class I and class II molecules on APC bind antigenic peptides derived from endogenous and exogenous proteins, respectively. Lipid antigens captured from exogenous antigens bind to CD1 MHC-like molecules.[37-38] Antigen processing and presentation by MHC class I[39-48] and CD1 MHC-like molecules[37, 49-50] have been described elsewhere; thus, they will not be described here.

T lymphocytes are derived from precursors in hematopoietic tissue and undergo cell differentiation in thymus. T cells expressing co-receptor molecule CD4 that recognize MHC class II-peptide complexes are called $CD4^+$ T cells (or helper T cells) and those expressing CD8 that recognize MHC class I-peptide complexes are called $CD8^+$ T cells (or cytotoxic T cells).[51-54] Upon activation, T cells can differentiate into different helper T cells, including T_H1 cells (IFN-γ and lymphotoxin producer),[3, 55] T_H2 cells (IL-4, IL-13, IL-5, and IL-6 cytokine producer),[3, 12, 55] or T_H17 cells (IL-17 producer).[56] Naïve $CD4^+$ T cells can also be differentiated into an independent lineage called regulatory T cells (T_{reg}) expressing fork-head transcription factor Foxp3 and CD25.[56] $CD4^+$ T cells execute many important effector functions, depending on the cytokines they secrete.[55] $CD8^+$ T cells are capable of differentiating into cytotoxic T lymphocytes (CTL);[10] these cells recognize specific antigens presented by MHC class-I on their target cells and perform cell lyses.[11, 13]

The MHC class II (MHC-II) molecules are expressed by professional APC (pAPC), including dendritic cells (DC), macrophages, and B cells. Each of these pAPC has different ways of capturing and processing antigens; for example, DC activate naïve T cells more efficiently than do B cells or macrophages.[57] Before the presentation of the peptide fragments of the antigen, the antigen is first internalized via phagocytosis, fluid-phase uptake by macropinocytosis, or receptor-mediated endocytosis via mannose and lectin-like receptors.[58-60] Then, they are processed via early endosome and then transferred to late endosome before entering lysosomes.[61-65] Because MHC-II compartments (MIIC) are rich in lysosomal enzymes, proteins are degraded by lysosomal proteases to generate peptide fragments; these peptides are loaded onto MHC-II molecules in the MIIC.[66-68] The new MHC-II molecules are synthesized and assembled with invariant chain (Ii) in the endoplasmic reticulum to form MHC-II-Ii complexes, which are then transported to the Golgi apparatus and targeted to the endosomal/lysosomal compartments.[69-72] The portion of Ii in MHC-II-Ii complex is then clipped off by proteases to produce MHC-II molecules with class II-associated invariant chain (CLIP). Then, the CLIP is exchanged for antigenic peptide to form the MHC-II-peptide (MHC-II-p) complex that is transported to the surface of the APC for presentation to a $CD4^+$ T cell.[48, 68]

The highly polymorphic MHC-II molecules consist of α- and β-chains, which belong to the immunoglobulin supergene family.[29] Each chain consists of two extracellular domains (α1 and α2; β1 and β2, respectively), a transmembrane domain, and a

cytoplasmic domain. The antigenic peptide binds to α1- and β1-domains.[73] Except for the α1 domain of MHC-II, all other extracellular domains possess an intradomain disulfide bond. The transmembrane and cytoplasimc domains of MHC-II posses specific sequences required for efficient T cell surface expression,[74] signaling,[75] antigen presentation,[76-77] and lateral diffusion of MHC-II molecules on the surface of the APC.[78]

1.1.3 Signal-2

Signal-2 or the co-stimulatory signal is necessary for amplifying and controlling the TCR signaling process, stabilizing the physical association, and regulating the T cell response.[79-80] The predominant co-stimulatory signal for T-cell activation is delivered by interaction between the B7 family of molecules on the APC and CD28 or CTLA-4 receptors on T cells (Fig. 2).[81-83] B7 co-stimulation along with TCR signaling generates the upregulation of α-, β-, and γ-chains of IL-12 receptor,[84-87] cytokine transcription,[88-89] T cell proliferation,[90-92] and expression of Bcl-χ_L.[93] During T cell-APC interaction, the absence of a co-stimulatory signal and the presence of TCR signal produce antigen-specific unresponsiveness (anergy) of T cells, which can be reversed with IL-12.[92, 94] Blocking the co-stimulatory signal (Signal-2) is the basis for designing therapeutic agents for inflammatory and autoimmune diseases. The balance between positive and negative signals delivered by CD28 and CTLA-4, respectively, is crucial for regulation of T cell proliferation, differentiation, and survival and T cell-dependent B cell responses. CD28 provides signals that augment and sustain T-cell activation in concert with TCR signaling. CTLA-4 (a negative regulator) antagonizes TCR signals to dampen secondary

immune responses for inducing tolerance. CD28 is expressed constitutively on all human $CD4^+$ T cells and on approximately 50% of human $CD8^+$ T cells.[95-96] Its level, however, increases after T-cell activation. In contrast to CD28, CTLA-4 is not expressed on resting T cells but is present on activated T cells as a late priming molecule and it is observed after 24–48 hours with only 2–3% of the levels of CD28 expression.[97] The expression of CTLA-4 in the immunological synapse is proportional to the strength of the TCR stimulus,[98] and it recruits *Src* homology 2 (SH2) domain-containing phosphatase-2 (SHP-2) upon T-cell activation[99] as a negative regulator of T cell responses.

B7-1 (CD80) and B7-2 (CD86) are the two members of the B7 family primarily expressed on APC.[90-91, 100] B7-1 is upregulated in response to maturational stimuli, while B7-2 is constitutively expressed at low levels and induced rapidly upon activation.[101] CD28 has lower avidity for B7-1 and B7-2 molecules than does CTLA-4.[102-103] Both types of B7 molecules exist as monomers with conserved residues at the extracellular V-like and C-type domains that are critical for binding to CD28 and CTLA-4 via the MYPPPY motif. B7-1 has a short cytoplasmic tail while B7-2 has a longer cytoplasmic tail with three potential phosphorylation sites by PKC.

Two other co-stimulatory signaling pathways are delivered using inducible costimulator (ICOS) and programmed death-1 (PD-1) molecules. ICOS molecule is a homodimer linked with a disulfide bond; it is a homolog of CD28 with a FDPPPF motif in the V-like domain instead of the MYPPPY motif. ICOS binds to B7h molecule, and its stimulation in response to TCR activation increases T cell proliferation[104-107] and promotes IL-10 production as well as increases IL-4, IL-5, IFN-γ, TNF-α, and GM-CSF

production.[105] In addition it plays an important role in tolerance induction.[108-109] Due to the absence of the MYPPPY binding motif, ICOS does not bind to B7-1 and B7-2.[107, 110] B7h is expressed constitutively by B cells and macrophages, and its expression can be induced by inflammatory stimuli on non-lymphoid cells, including endothelial cells, fibroblasts, and epithelial cells.

The PD-1 molecule has roles in induction and/or maintenance of peripheral tolerance and acts as a negative regulator of T cell responses. Functional studies showed that PD-1-deficient Balb/c mice developed a fatal dilated cardiomyopathy with early disease onset,[111] The ligands for PD-1 are PD-L1 (Fig. 2) and PD-L2. PD-L1 is widely expressed on T cells, B cells, macrophages, monocytes, DCs, and non-lymphoid cells, including endothelial cells, syncytiotrophoblasts in the placenta, muscles, and pancreatic islets,[113] while PD-L2 is only found on macrophages and DCs inducible by cytokines.[114] PD-1 is expressed during thymic development primarily on $CD4^-CD28^-$ and $\gamma^-\delta^-$ thymocytes and induced on peripheral $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes upon TCR stimulation.[113] Unlike CTLA-4, PD-1 inhibits expression of the cell survival gene bcl-X_L,[113] and both CTLA-4 and PD-1 are known to limit glucose metabolism and Akt activation via different pathways. CTLA-4 inhibits Akt activation via protein phosphatase 2a, and PD-1 inhibits via CD28-mediated activation of PI3K.

LFA-1 and ICAM-1 are accessory molecules, which have adhesion and/or co-stimulatory functions (Fig. 2). LFA-1 is a well-established cell-cell adhesion molecule; it plays a key role in several of T-cell activation and effector function.[115] Functionally, LFA-1 has been shown to enhance IL-2 expression, leading to the induction of T cell

proliferation. LFA-1 is exclusively expressed on leukocytes and interacts with its ligands such as ICAM-1, -2, and -3 to promote a variety of homotypic and heterotypic cell-cell adhesion necessary for normal and pathologic functions of the immune systems. The interaction of LFA-1 with its ligands permits allows for improved adhesion of leukocytes to vascular endothelium, an essential step in the recruitment and migration of leukocytes into inflamed tissue.[116-118] Similarly, LFA-1-mediated adhesion is thought to facilitate antigen presentation to T cells, and numerous reports indicate that LFA-1 engagement decreases the minimal stimulatory dose of antigen by 10- to 100-fold.[119-121]

1.1.4 Signal-3

After Signal-1 and -2 have been initiated, inflammatory cytokines are released in order to fully activate the T cell; this is known as Signal-3.[122] The inflammatory cytokines can be released by the APC (paracrine) or by the T cell itself (autocrine)[123] and the cytokines are required for the proper proliferation, differentiation, and activation of T cells. This signal is also called the "polarizing" signal because after its delivery, helper T cells become polarized into either type 1 helper T cells (T_H1) or type 2 helper T cells (T_H2).[124] Matzinger proposed the "danger theory," which states that, for full activation, the T cell must receive a "danger" signal provided by cytokines and co-stimulatory signals.[125-126] Matzinger believed that the immune system was driven by its power to recognize danger and deploy an immunizing response that will remove the threat and prevent harm to the body.[125] If the "danger" signal is not delivered, the immune system will induce a tolerizing effect rather than an immunizing effect.

To study how the "danger" signal affected the proliferation of $CD8^+$ and $CD4^+$ T cells, Mescher *et al.* employed artificial APC, using microspheres coated with immobilized MHC protein/peptide complexes to provide Signal-1 and co-immobilized B7-1 protein for Signal-2.[122] For $CD8^+$ T cells, they proved that IL-12 was needed along with Signal-1 and Signal-2 to stimulate naive $CD8^+$ T cells for optimal proliferation. Also, they showed that without IL-12, the T cells do not differentiate into cytolytic effector cells and thus they are not fully activated. In contrast, IL-1 but not IL-12 was required for the optimal proliferation of naïve $CD4^+$ T cells. Thus, inflammatory cytokines produced by APC are the necessary Signal-3 to optimize the proliferation of $CD4^+$ and $CD8^+$ T cells to prevent tolerance toward a specific antigen.

As a "polarizing" signal, Signal-3 is delivered to naïve helper T cells for their differentiation to either T_H1 or T_H2, and the differentiation depends on the type of cytokine delivered as the Signal-3. If the naïve helper T cells receive IL-12 signal, they differentiate into T_H1. If they receive IL-4, the helper T cells differentiate into T_H2.[124] Once the helper T cells are differentiated, T_H1 cells secrete cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which are used to direct T cell immunity against intracellular bacteria and viruses. If the naïve T cell differentiates into T_H2 cells, their job is to control humoral immunity and immunity against extracellular parasites by producing cytokines such as IL-4, IL-5 and IL-13.

T cells can produce Signal-3 for optimization of their own proliferation. After the delivery of Signal-1 and Signal-2, transductions of several intracellular signals such as

the calcium-calcineurin pathway, the RAS-mitogen-activated protein (MAP) kinase pathway, and the nuclear factory-κB pathway (NF-κB) are generated.[123, 127] These pathways activate expression of the most prominent cytokine, IL-2, along with several other cytokines (Fig. 2). Binding of IL-2 to IL-2 receptor (IL-2R) on the surface of the T cells followed by other cytokines initiates T cell proliferation via the rapamycin pathway.[123] Although cytokines are very important for inducing the appropriate T cell response, it is still unclear where and how most of these inflammatory cytokines act to set off T cell responses in both $CD4^+$ and $CD8^+$ T cells.[122]

1.2 Antigenic Peptides

Autoimmune diseases could involve a single organ (e.g., multiple sclerosis, rheumatoid arthritis, and diabetes) or no involvement of specific organs (e.g., systemic lupus erythematosus). T cells and B cells are engaged in inflammatory immune responses, leading to the onset and progression of autoimmune diseases. Activation of both T and B cells depends on antigen presentation. Although antigens are necessary for activation of these lymphocytes, several studies have shown that injections of antigenic peptides/proteins could also suppress autoimmune diseases. The mechanisms of suppression by antigens are thought to work by immune deviation and induction of T_{reg} cells. In the following sections, etiologies and current treatments of various autoimmune diseases are discussed.

1.2.1 Multiple Sclerosis

Multiple sclerosis (MS) is a complex genetic inflammatory disease in which the autoreactive immune cells (i.e., T cells) attack the myelin sheath of the central nervous system causing axonal damage, demyelination (Fig. 3), and chronic inflammation (Fig. 3).[128] As a result of myelin sheath damage, the communication between nerve cells is disrupted due to interruption of the conduction of the electric impulses along the axons. In MS patients, the damage in the myelin sheath affects optic nerves, brainstem, spinal cord, cerebellum, and periventricular white matter. The formation of lesions or plaques called sclerosis in the white matter of the brain and the spinal cord are the pathological hallmark of the disease. Besides the destruction of the myelin sheath, the lesions are also due to the loss of axons and the death of oligodendrocyte cells, which are responsible for the formation and maintenance of the myelin sheath. As a result, MS patients suffer from muscle weakness, changes in sensation and speech, vision problems, muscle spasms, and fatigue.

Nearly 80% of MS patients have clinical relapses referred to as relapsing-remitting MS (RRMS), which is characterized by inflammation of the brain and spinal cord along with white matter lesions.[129] During the RRMS period, patients have unpredictable relapses followed by long remission with no signs and symptoms of the disease; about 65% of these patients will gradually progress toward a secondary progressive MS (SPMS) typified by an irreversible neurological defect.[129] About 20% of the patients without remission have steady neurological decline from the onset of the disease and are categorized as having primary progressive MS (PPMS), which is the least common type.

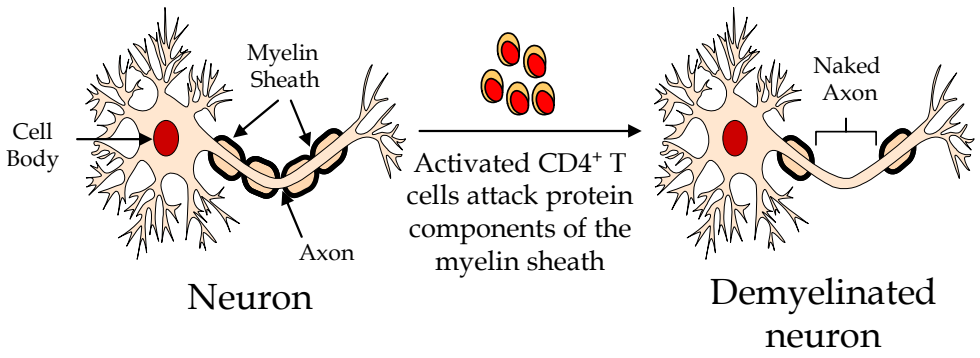

Figure 3. Cartoon representation of a healthy neuron and a demyelinated neuron.

Both PPMS and SPMS show signs of axonal degeneration, which could be due to loss of the myelin sheath.

The cause of MS has not been fully elucidated; however, it is clear that MS patients have a leaky blood-brain barrier (BBB) with brain infiltrations of $CD4^+$/$CD8^+$ T cells, B cells, and monocytes.[128, 130-131] Although the antigenic target of the immune cells is not certain, there is increasing evidence that the T cells recognize self myelin antigens such as proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or antigenic peptides from the immunodominant epitopes of MBP (MBP_{84-104}), MOG (MOG_{35-55} and MOG_{92-106}), or PLP ($PLP_{139-151}$ and $PLP_{178-191}$) (Table 1). Once activated with myelin antigens, naïve $CD4^+$ T cells migrate into the CNS where these cells recognize the antigen presented by the local APC called microglia. Naïve $CD4^+$ T cells then undergo a process of differentiation into T_H1 effector cells, which produce proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α.[132] In addition to T_H1 subset activation, a subpopulation of memory $CD4^+$ T cells (T_H17) are also activated; these cells have been found to secret the proinflammatory cytokine IL-17 under the control of IL-6, IL-23, and TGF-β.[133-134] An elevated level of IL-17 in the blood and CSF has been observed in MS patients.[135-136] Circulating IL-17 and -22 are responsible for disruption of the BBB, which eventually allows T_H17 cells to pass through the BBB to damage the neuronal cells.[129] The experimental autoimmune encephalomyelitis (EAE) mouse has been used as a model for MS. Injections of anti-IL-17 monoclonal antibody (mAb) after EAE induction in mice prevents the development of the disease, suggesting that IL-17 has an important role in the pathogenesis of EAE and MS.[134, 137]

Table 1. List of candidate autoantigens in MS, T1D, and RA		
Disease	**Antigen**	**References**
Multiple Sclerosis	Proteolipid protein (PLP) Myelin basic protein (MBP) Myelin oligodendrocyte glycoprotein (MOG) Astrocyte-protein S100β	314
Type 1 Diabetes	Glutamic acid decarboxylase (GAD) Insulin zinc transporter (ZNT8) Insulinoma antigen (IA-2) Insulin	161-163
Rheumatoid Arthritis	Human cartilage gp-39 Cartilage proteins melanoma inhibitory activity (MIA) Type II collagen Citrullinated proteins (e.g., fibrin)	315-321

Several studies have shown that T cell-mediated immune response to myelin antigens can be tolerated by administrating autoantigens. Oral administration of MBP to Lewis rats prior to the induction of EAE with MBP and complete Freund's adjuvant has shown to suppress EAE as a result of clonal anergy or deletion of activated T cells.[138-141] This observation was further supported by a reduction in the number of T_H1 MBP-specific cells in tolerance-induced rats.[141] A one-year, double-blind, clinical study of a small group of MS patients administered bovine myelin antigens showed a significant decrease in the number of T cells reactive toward myelin basic protein compared to the control group.[142] In this study, a significant decrease was observed in the number of patients undergoing major attacks (6/15) compared to the control group (12/15).[142] However, a subsequent large, phase III clinical trial failed to confirm the efficacy of a single oral dose of myelin antigens (MBP and PLP) due to a large placebo effect. This study has left many unanswered questions about the dose or formulation of myelin that might have been more effective.

Alternatively, intrathecal, intravenous (i.v.), subcutaneous (s.c.), or nasal administration of antigenic peptides has been investigated to induce tolerance in patients. Two phase I trials conducted by Warren and colleagues demonstrated that administration of MBP peptides, MBP_{75-95} or MBP_{85-96}, in chronic progressive multiple sclerosis patients did not produce any adverse effects.[143-144] Induction of tolerance was monitored by detecting the reduction of anti-MBP antibodies in cerebrospinal fluid (CSF). Intrathecal administration resulted in transient neutralization whereas intravenous administration resulted in long-lasting tolerance or reduction in anti-MBP antibodies for up to one year

after a second i.v. dose of antigen. However, s.c. administration of the antigenic peptides did not affect the reduction of anti-MBP antibodies in CSF, clearly demonstrating that s.c. injection failed to induce tolerance in these patients.[144] Unfortunately, these studies failed to relate the clinical significance of the reduction in antibodies against MBP and its effect on the prognosis of the disease in MS patients.

Antigenic peptides derived from myelin basic protein were successfully delivered via the nasal route and were effective in inhibiting EAE disease in a murine model.[145-148] The mechanism of tolerance in this model is due to MBP-reactive specific T_{reg} cells that produce IL-10. In addition, these cells were found to express higher levels of IL-4 and TGF-β mRNA.[147, 149] Similarly, altered peptide ligands (APL) are mutated antigenic peptides with increased affinity for MHC-II and/or TCR, and they have been shown to control rheumatoid arthritis and EAE in animal models. A suggested mechanism of action for APL in inhibiting antigen-specific T-cell activation is increasing the expression of indoleamine 2, 3-dioxygenase (IDO) enzyme in activated T cells. Presumably, the increased amount of IDO converts tryptophan to its metabolites (i.e., 3-hydroxykynurenic acid (3-HKA), picolinic acid (PA), and quinolinic acid (QA)) and these metabolites suppress the EAE relapse by reducing the production of inflammatory cytokines and stimulating the production of IL-4 and IL-10 that are related to T_H2 and T_{reg} cellular differentiation.[150]

Current strategies that are approved for the treatment of MS include two classes of drugs, these are anti-inflammatory and disease-modifying therapies (DMT). Acute MS relapses are often treated with high-doses of intravenous corticosteroids such as

methylprednisolone. This is effective for short-term treatment, but it does not have any effect on long-term recovery. Mitoxantrone (Novantrone®), interferon beta-1a (IFNβ-1a or Avonex®/Rebif®/CinnoVex®), IFNβ-1b (marketed as Betaseron®/Betaferon®), glatiramer acetate (GA, Copaxone®), and natalizumab (Tysabri®) are current DMT drugs. Mitoxantrone will not cure the disease, but it is effective in slowing the progression of SPMS and delaying the relapse interval time in RRMS.[151] The mechanism of action of mitoxantrone involves disrupting both DNA synthesis and DNA repair. The side effects of mitoxantrone are irreversible cardiomyopathy, acute leukemia, and bone marrow suppression; patients receiving this treatment are advised to have regular echocardiograms.

IFNβ-1b was first introduced in 1993 for RRMS treatment. In the clinical trials, patients treated with IFNβ-1b were free from relapse at the end of two years compared to a placebo-treated group.[152] IFNβ-1b acts as an anti-inflammatory agent by reducing the production of IFN-γ and TNF-α and inhibits T-cell activation, clonal expansion, and migration into the CNS.[153] The mechanism of action of IFNβ-1a is thought to be similar to that of IFNβ-1b.

Copaxone® (glatiramer acetate) is a synthetic peptide polymer (40–100 residues) that consists of Glu, Lys, Ala, and Tyr amino acids in a ratio of 3:7:9:2; it is designed as a decoy for myelin basic protein (MBP). Copaxone® has been suggested to alter the immune response from T_H1- to T_H2-cell differentiation.

Finally, natalizumab (Tysabri®) is the first recombinant human monoclonal antibody (mAb) approved for the treatment of MS. It inhibits leukocyte adhesion to vascular

endothelium by binding to the α_4-subunit of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins on the surface of leukocytes to prevent leukocyte infiltration into the CNS.[154] Natalizumab has been shown to significantly slow the progress of disability with increasing numbers of disease-free individuals compared to placebo group. It can also reduce disease relapse, loss of vision, and number of lesions in patients.[155-157] In spite of its greater efficacy compared to any other approved MS therapies, its use is limited because of the development of potential fatal adverse effects such as progressive multifocal leukoencephalopathy (PML), melanoma, hepatotoxicity, opportunistic infections, and primary CNS lymphoma.[158-159] Recent studies suggest that patients receiving this drug for more than one year are at increased risk for PML.[160] Currently, the FDA has limited the usage of natalizumab to patients not responding to other therapies, and the drug is under a mandatory safety monitoring program.

1.2.2 Type-1 Diabetes

Type 1 diabetes (T1D) is marked by insulitis and the destruction of the pancreatic islet of Langerhans beta cells.[161] Although not all-inclusive, studies carried out in non-obese diabetic (NOD) mice show that $CD4^+$ and $CD8^+$ T cells are partly responsible for the destruction of the β-cells.[5] Those afflicted with T1D are genetically predisposed, and the progress toward the disease is initiated after encountering an environmental insult, such as diet or microbial infections.[5] Insulin, glutamic acid decarboxylase (GAD), zinc transporter (ZNT8), and insulinoma antigen (IA-2) are the four major antigens (Table 1) for activation of immune cells, and they have been used in assays to detect the progress

of T1D.[162-163] Glutamic acid carboxylase 65 kDa (GAD65) is considered a target for therapy because of its partial role in the activation of certain T cells that recognize it as an antigen.[161]

The immunotherapy of T1D focuses on two alternative methods of treatment: prevention of T1D or treating it at its onset. Ever since its discovery, insulin has been considered to be the gold standard of treatment for Type 1 diabetics. Like GAD65,[161] insulin has also received attention as a therapeutic method to suppress the activation of T cells.[163] By suppressing the activation of T cells, beta-cells may be regenerated, and this may lead to the remission of diabetes.[5] The administration of autoantigens (GAD65 or insulin) and their peptides has been shown to suppress beta-cell autoimmunity.[5, 161]

The role of insulin as an autoantigen is still ambiguous. To date, many epitopes of insulin and proinsulin have been identified.[163] Recent findings have shown that insulin autoantibodies can be induced by insulin B9-23 peptide. NOD mice (H-2d) were immunized with B9-23 peptides, which led to the development of MHC-activated autoantibodies towards insulin.[164] However, it has been shown that the antibodies toward insulin react only with insulin and not with NOD B9-23 peptides, suggesting that insulin antibodies recognize the whole molecule rather than the peptide sequence itself.[163] Proinsulin, the precursor of insulin, also has a sequence of amino acids (B24-C36) that are recognized by NOD mice $CD4^+$ T cells.[165] B9-23 has been hypothesized to play an important role in humans as it did in NOD mice because the amino acid sequences are similar in both species. Higashide and colleagues have shown that the most frequent epitopes recognized by T cells are B10-24, B1-15, and B11-25, with B9-23, B4-18, and

B12-26 being identified in some patients.[166] Alternatively, it has been observed in NOD that intranasal vaccines containing proinsulin in a plasmid DNA increased the number of T_{reg} cells and effectively prevented the onset of diabetes.[167]

GAD65 is an enzyme that is targeted as an autoantigen by T cells. Anti-GAD65 antibodies can be observed in NOD mice prior to the onset of T1D. Injection of GAD65, intravenously or intrathymically, to young NOD female mice prior to any signs of disease has been shown to prevent diabetes and induce $CD4^+$ T_{reg} cells.[168] Controlling the activation and increasing the induction of T_{reg} cells by GAD65 antigen may help suppress T1D.[161] Tisch and colleagues revealed that peptides derived from GAD65 are able to suppress the progression of T1D in NOD mice prior to the onset of insulitis. The peptides used include amino acids: 217-236, 247-265, 290-309, and 524-543.[169]

1.2.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic idiopathic disease characterized by persistent inflammation and local destruction in the synovial tissues including the synovium, bone, and cartilage. Although the nature of the antigens responsible for RA pathogenesis has not been clearly elucidated, there is evidence that the disease-associated HLA DR (B1*0401, 0404, 0405, and 0101) molecules, which reside in the MHC and participate in antigen presentation, are associated with the disease.[170] Viral proteins such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) as well as several autologous proteins normally expressed in the joints, including gp39, proteoglycan, and type II collagen, have been implicated in the generation of the pathogenic T cell response in RA

(Table 1). The driving force behind rheumatoid inflammation is believed to be $CD4^+$ T cells responding to an antigenic epitope in the synovium in an HLA-DR-restricted manner. Increased numbers of "memory" $CD4^+$ and decreased numbers of "naïve" $CD4^+$ T cells in the synovial tissues of RA patients[171-172] suggest that $CD4^+$ T cells play a crucial role in the pathogenesis of RA. Synovial tissue T cells are enriched with $CD4^+$ T cells that do not express the Leu 8 antigen, now known to be the human equivalent of the murine homing receptor.[173-175] Loss of this molecule is characteristic of activated T cells. Synovial fluid also contains augmented expression of the activated antigens HLA-DR, CD9, CD38, CD49a/CD29 (VLA-1), and adhesion molecule CD54 (ICAM-1).[176-179]

In addition to $CD4^+$ T cells, a number of other inflammatory mediators produced in the rheumatoid synovium, including arachidonic acid metabolites, vasoactive amines, platelet-activating factor, and complement cleavage products contribute to the inflammation. Recently IL-17 produced primarily by the T_H17 cells has also been implicated in human autoimmune inflammation. A potential key role in diseases such as RA has only recently been attributed to IL-17, although it was discovered decades ago. Elevated levels of IL-17 in blood and synovium of RA patients have been correlated with synovial levels and joint damage.[180-182] Recent advancement in research toward new and better-tolerated therapies to attenuate the inflammation and pain associated with rheumatoid arthritis as well as halt the progression of erosive joint damage has led to the development of strategies for modulating stimulation of immune cells or blocking cytokines as potential therapeutic strategies.

Injections of soluble antigens have been shown to induce antigen-specific immune tolerance. It is thought that tolerance could be achieved through anergy/deletion of $CD4^+$ T cells or the induction of $CD4^+$ T_{reg} cells that produce IL-10 or TGF-β.[183] Oral and, more recently nasal, tolerance resulting from administration of antigens has attracted attention as a potential treatment of autoimmune diseases. Oral administration of type II collagen (CII) has been shown to suppress arthritis in mice[184] and rats.[185] Oral administration of chicken CII to patients with RA provided significantly less progression of the disease compared to placebo-treated patients.[186]

In another study, peripheral blood mononuclear cells (PBMC) of RA patients responded well *in vitro* to $CII_{256-271}$ epitope and its overlapping variants.[187] Similarly, oral administration of this peptide has been shown to suppress arthritis in a CIA mouse model.[187] Specific T cell activity, proliferation, and secretion of IFN-γ in spleen cells were actively suppressed in $CII_{250–270}$-fed mice, and the serum anti-CII, anti-$CII_{250–270}$ antibody activities and frequency of specific antibody-forming spleen cells were significantly lower in $CII_{250–270}$-fed mice than in controls. Moreover, IL-4-producing cells (T_H2 cells) were upregulated when CIA mice were treated with type II collagen ($CII_{250-270}$), suggesting that oral administration of $CII_{250–270}$ can suppress the cellular and humoral immune response in collagen-induced arthritis. Although the majority of animal studies have yielded positive results with the oral tolerance regimen, under some circumstances, mucosal application of antigen may instead exacerbate the disease process.[188]

Abatacept, also known as CTLA4-Ig, was approved in 2005 by the FDA for the treatment of patients with moderate-to-severe active RA who have had an inadequate response to disease-modifying antirheumatic drugs (DMARDS), including methotrexate (MTX) and TNF antagonists. It is a fusion protein composed of the extracellular domain of the CTLA4 molecule complexed to the Fc domain of human IgG1. The IgG1 solubilizes the fusion protein; thus, unlike the membrane-bound CTLA4 molecule, its binding to CD80/CD86 does not induce a negative signal to T cells. Its binding blocks the engagement of the CD28 co-stimulatory signal required for T-cell activation, resulting in T cell anergy.[189] Patients receiving abatacept responded better than those receiving placebo. Quality of life was also improved with abatacept and benefits were sustained at 12 months, these included significant reduction in disease activity as well as improved physical function.[190]

Infliximab is a chimeric monoclonal antibody that binds to circulating and transmembrane TNF-α and thereby neutralizes its biological effect. Circulating TNF-α is a form of TNF-α generated by cleavage from the transmembrane precursor. Both forms of TNF-α have been shown to sustain the inflammation process. Transgenic mice overexpressing TNF-α were observed to develop a destructive arthritis resembling rheumatoid arthritis (RA). Moreover, high levels of TNF-α detected in synovial fluid and in the tissues of patients with RA[191] suggest that TNF-α plays a pivotal role in physiologic and pathogenic response.[192] In addition to infliximab, a number of agents that block TNF-α have been developed for clinical use, including etanercept, adalimumab, and certolizumab. Etanercept is a fusion protein consisting of the soluble human TNF-α

receptor linked to the Fc-portion of the human IgG1. Adalimumab is a recombinant human IgG1 monoclonal antibody specific for TNF-α; it not only inhibits binding of TNF-α to its receptors but also lyses cells expressing membrane-bound TNF-α. Certolizumab, on the other hand, is a polyethylene glycol (PEG)-conjugated humanized anti-TNF Fab fragment. Although anti-TNF-α drugs have been shown to be effective and relatively safe for use in treating RA, blockade of this cytokine may have effects beyond the suppression of synovial inflammation, which can influence mortality of these patients. Mycobacterial infections have been reported among patients undergoing therapy with infliximab.[193-194] Therefore, tuberculin skin tests and a chest radiograph are usually recommended before initiation of anti-TNF treatment. Also, avoidance of anti-TNF therapy is recommended for patients with severe chronic heart failure.[195] Aside from TNF-α, cytokines appear to be of particular importance in the development of RA and, therefore, have been investigated as therapeutic targets. Kineret (Anakinra) is a recombinant, nonglycosylated form of the IL-1 receptor antagonist (IL-1Ra), a naturally occurring receptor antagonist known to counteract the proinflammatory effects of interleukins. Patients taking background MTX with anakinra had significant clinical improvement as compared to the placebo group.[196-197] Simultaneous blockade of TNF-α and IL-1 receptor had very promising results in experimental murine arthritis;[198] however, this did not translate well to clinical outcome. Moreover, the risk of mycobacterial infections under combination therapy increased significantly; it is therefore not recommended for RA treatment.[199] Tocilizumab (MRA) is a humanized monoclonal antibody against IL-6 receptor (IL-6R). Randomized phase II studies showed

that patients receiving tocilizumab had reduced disease activity in a dose-dependent manner.

B lymphocytes play several critical roles in the pathogenesis of rheumatoid arthritis. They are the source of rheumatoid factors and anticitrullinated protein antibodies, which contribute to the immune complex and complement of T-cell activation in the joints. It is therefore assumed that the depletion of B lymphocytes provides a potential therapeutic approach to controlling disease activity and inducing remission. This hypothesis has been proven in an open-label study of rituximab, a chimeric monoclonal antibody against the protein CD20. CD20 is widely expressed on B cells from early pre-B-cells to later stages in differentiation, but it is absent on terminally differentiated plasma cells. Rituximab was significantly superior to placebo when added to MTX.[200] Safety and tolerability of rituximab were found to be comparable to that of the placebo. Moreover, there have been no higher infection rates under B cell depletion therapy.[200]

1.3 Blocking Signal-2

Blocking Signal-2 formation has been shown to alter the immune response via induction or anergy of a certain T cell phenotype (i.e. T_H1 or T_H2). Inhibition of Signal-2 has been used for the treatment of autoimmune diseases. Monoclonal antibodies and peptides (Table 2) that inhibit Signal-2 have been shown to suppress allograft rejection,[201-202] type-1-diabetes,[203-204] rheumatoid arthritis,[205-206] and psoriasis.[207-208] Despite the great promise of mAb, these drugs are costly to produce and difficult to formulate or deliver. Peptides and peptidomimetics are a better alternative to mAbs.

Table 2. Antibodies and peptides that have been investigated for blocking Signal-2 in autoimmune diseases, organ transplantation, and cancer			
Signal-2 Receptor	Peptide/Protein Ligand	Target Disease	References
LFA-1/ ICAM-1	Anti-LFA-1 mAb (Odulimomab and Efalizumab)	Psoriasis, diabetes, and transplantation	213, 215-218, 227-228, 242
	Anti-ICAM-1 mAb (Enlimomab)	Rheumatoid arthritis, diabetes, and transplantation	227, 236-237
	Cyclo(1,12)-PenPRGGSVLVTGC (cIBR)	Rheumatoid arthritis, inflammation, and immunosupression	250, 322
	Cyclo(1,12)-PenITDGEATDSGC (cLABL)	Rheumatoid arthritis, inflammation, and immunosupression	250, 252-253
	Cyclo(1,9)-CLLRMRSIC	Inflammation	323
B7/CD28	Anti-CD28 mAb	Transplantation	324
B7/CTLA-4	Anti-CTLA4 mAb (Ipilimumab and Tremelimumab)	Melanoma and other types of malignancies	292
	Abatacept (CTLA4-Ig)	Rheumatoid arthritis	189

Despite progress in designing small molecule inhibitors, a limited number of small molecules have been designed to target Signal-2, but none has yet reached the clinical setting.

1.3.1 LFA-1/ ICAM-1 interaction

Studies performed *in vivo* and *in vitro* have demonstrated that T-cell activation requires interaction of adhesion receptors. Initially, when T cell, encounter APC, adhesion receptors physically anchor and provide feedback to the T cells about the surface milieu of the APC. This may indeed be followed by TCR signaling and tightening of the distance between the two membranes through translocation of the receptors.[6, 209] Adhesion receptors such as ICAM-1 are considered to be involved in intracellular signaling, leading to the accumulation of the immune receptors along with MHC molecules at the contact area for the efficient presentation of the MHC-p complex to the T cells.[210] Thus, adhesion is considered a co-stimulatory signal required to generate TCR signaling and, further, immune response. There are several adhesion receptors on T cells that can deliver a co-stimulatory signal including LFA-1. LFA-1 interacts with ICAM-1, -2, and -3.[210-211]

Efforts made toward understanding the structure, function and mechanisms of interaction between LFA-1 and ICAM-1 have produced possibilities of new therapies. Currently, several strategies have been developed using LFA-1 and ICAM-1-targeted therapeutics, which include antibodies, peptides, peptidomimetics, small molecules, and antisense oligonucleotides to suppress LFA-1/ICAM-1 co-stimulatory signal for the

treatment of inflammation,[212] autoimmune diseases,[213] allograft rejection,[214-219] and cancer.[220] The mechanism of action for these antagonists may be very complex. It may include binding of the antagonists to either LFA-1 or ICAM-1 for blocking their interaction. The antagonist could inhibit or down-regulate the surface expression of the receptor or inhibit activation of the receptor.

1.3.1.1 Antibodies

The role of ICAM-1 and LFA-1 in disease has been studied extensively using anti-ICAM-1 and anti-LFA-1 monoclonal antibodies. The therapeutic effectiveness of these antibodies has been demonstrated in numerous animal models of transplant,[221-224] arthritis,[225] insulin-dependent diabetes mellitus,[226-229] multiples sclerosis,[230-234] and lupus.[235] The first LFA-1/ICAM-1-targeted monoclonal antibody tested clinically was anti-LFA-1 antibody Odulimomab (antibody 25.3) in bone marrow[215, 218] and kidney transplant,[216-217] while anti-ICAM-1 antibody Enlimomab (antibody R6.5 or BIRR1) has been investigated in kidney transplant[236] and rheumatoid arthritis.[237] Anti-ICAM-1 antibody is progressing through phase I and II trials for the treatment of rheumatoid arthritis.[238] Blocking ICAM-1/LFA-1 interaction using Enlimomab failed to show any benefit in a randomized renal transplantation study.[239] A recombinant humanized monoclonal antibody that binds to CD11a (the α-subunit of LFA-1), efalizumab has been successfully used in the treatment of psoriasis.[213, 240-247] Recently, a crystal structure of the efalizumab Fab in complex with the LFA-1 I-domain reveals that the antibody binds to the I-domain distinct from the ICAM-1 binding site and blocks the binding of LFA-1

to ICAM-1 via steric hindrance.[248] Efalizumab, under the trade name Raptiva® has been approved by the FDA in 2003 for the treatment of moderate-to-severe plaque psoriasis, it has been withdrawn from the market because of increased patient susceptibility to PML.[249] Although monoclonal antibodies such as Efalizumab successfully demonstrated their ability to induce tolerance, increased risk for secondary infections, immunogenicity, and lesser clinical efficacy limited their therapeutic use. Hence, alternative molecules such as peptides and small molecules are being developed for their utility in immunotherapy; these offer distinct advantages in terms of safety and development and treatment costs.

1.3.1.2 Peptides

The Siahaan group has discovered several cell adhesion peptides that block LFA-1/ICAM-1 interaction (Signal-2). These peptides were derived either from domain-1 of ICAM-1 or α- and β-subunits of LFA-1. The peptides derived from ICAM-1 and LFA-1 are listed in Table 2. Studies done using *in vitro* cellular models such as homotypic or heterotypic T cell adhesion or mixed lymphocyte reaction have clearly demonstrated the ability of these peptides to inhibit the binding of T cells to APC by blocking the binding of adhesion receptors to their natural ligands.[250-256] However, *in vivo* administration of these molecules for suppression of autoimmune diseases without general immune suppression is absolutely critical, as blocking Signal-2 may also create a general suppression of T-cell activation that may lead to increased susceptibility to secondary infections.

1.3.1.3 Antisense oligonucleotides

ICAM-1 is constitutively expressed by several cell types including endothelial and epithelial cells and is upregulated during many pathological conditions such as inflammation and autoimmune diseases. Alicaforsen (ISIS 2302), an antisense oligonucleotide, has been designed to specifically block ICAM-1 synthesis. Alicaforsen binds to the RNA molecule and further blocks the translation of the ICAM-1 protein within the cell.[257-258] The clinical outcome of Alicaforsen was assessed in several randomized controlled phase II and III trials for the treatment of Crohn's disease and ulcerative colitis. Initial trials involving a small group of subjects have shown promising results for both diseases, but later larger trials failed to meet the clinical efficacy tests.[259-265] Similar results were obtained in the rheumatoid arthritis study, and no significant difference was found between placebo- and ISIS 2302-treated groups, even though the drug was well tolerated.[266]

1.3.2 CD28/B7 interaction

CD28/CTLA-4:B7 interactions (Fig. 2) provide critical co-stimulatory signals required for complete T-cell activation. Molecules designed to block CD28/CTLA-4:B7 interactions have been widely investigated for immunotherapeutic strategies to regulate autoimmune diseases as well as response to transplantation.[267-270] It has been suggested that inhibition of the co-stimulatory signal via the CD28 pathway is more desirable because CTLA-4:B7 interactions have been shown to participate in the extinction of the T-cell receptor-mediated activation signal and induction of immunotolerance. The signal

transmitted through CTLA-4 leads to dephosphorylation of the second messengers in the CD3 complex and subsequently leads to control of the production of various cytokines produced by T_H1 and T_H2 cells.[271-272] Moreover, CTLA-4 has been implicated in the development of T_{reg} cells in several models of organ transplantation,[273] and CTLA-4-deficient animals have been shown to be resistant to immunotolerance.[274] However, selective CD28 inhibition decreases the activation of alloreactive and autoreactive T cells but not the activation of T cells stimulated by exogenous antigens presented in the context of self MHC. Antibody-induced modulation of co-stimulatory signal via CD28 pathway has been shown to prolong allograft survival in rats.[275] Similarly, administration of anti-CD28 Fab fragments efficiently led to suppression as well as reversal in the induction of EAE[276] and uveoretinitis[277] in mice. In CD28-deficient mice, immune responses to viral antigens or autoantigens are impaired,[278-279] whereas responses to exogenous antigens remain normal,[280] suggesting that T cell responses to autoantigens or alloantigens are more dependent on the costimulation through CD28 than on T cell responses to exogenous antigens. Thus, blockade of CD28 may be a better target for immunosuppression for the selective inhibition of pathologic T cells in autoimmunity and transplantation without inhibition of other protective T cell responses. CD28 blockade cannot be achieved with anti-CD28 dimeric antibodies because of target clustering, which promotes T cell costimulation.[281-282] So far, all antibodies investigated against human CD28 are indeed agonists. Conversely, monovalent fragments can block CD28/B7 interactions without stimulating CD28[282] but, in spite of all this potential, fab fragments cannot be used therapeutically *in vivo* because of their rapid elimination from the body.

Conjugation to both polyethylene glycol and molecular fusion with serum albumin, however have been investigated to improve the stability of these molecules and thereby extend their half-lives in the body.

Several studies have shown that selective blockade of CD28:B7 interaction using anti-CD28 Fab administration significantly ameliorated EAE.[276, 283] It is thought that amelioration could be largely or in part due to ablation of TNF-α production.[276] TNF-α has been associated with encephalitogenicity.[284-285] It is thought that T cell infiltration into the CNS, together with TNF-α inhibition, led to suppression of EAE.[285] However, results from recent studies have shown that EAE can be induced in mice deficient in lympotoxin-α and TNF-α, suggesting that other cytokines can replace their central pathogenic role in EAE.[286] Although CD28 blockade using mAb attenuated EAE and prevented subsequent relapses, it did not completely eliminate the encephalitogenic response, suggesting that activation of encephalitogenic T cells independent of CD28 costimulation may not have been completely abolished. This observation is consistent with findings in CD28-deficient mice. CD28-/- mice developed autoimmune heart disease, although it was less severe than that observed in heterozygous littermates.[278] Similarly, breeding NOD mice with CD28-/- was found to exacerbate autoimmunity.[287] All these taken together suggest that inhibition of CD28 costimulation using anti-CD28 Fab and suppression of autoimmunity may be at least partially dependent on T cell costimulation. It is also plausible that CD28 inhibition may prevent further recruitment and activation of naïve T cells and therefore interfere with epitope spreading.

1.3.3 CTLA-4/B7 interaction

Anti-CTLA-4 monoclonal antibodies can block the interaction between CTLA-4 and B7, there by blocking the inhibitory signal sent by CTLA-4. It is hypothesized that this blockade improves the ability of cytotoxic T cells to respond better to antigen presentation by APC, thus enhancing the activation and proliferation. In this section, several studies conducted on the effect of anti-CTLA-4 on autoimmune diseases such as EAE and lupus will be discussed as well as the efficacy of anti-CTLA-4 mAb treatment in tumor therapy.

The effects of inhibiting the CTLA-4/B7 interaction were studied in the mouse EAE model using hamster anti-mouse CTLA-4 mAb.[288] This study has shown that a single injection of anti-CTLA-4 two days post-immunization in mice resulted in a mild increase in severity and incidence of EAE. In another experiment, anti-CTLA-4 was administered on the second day after clinical signs of EAE were observed and then every other day for the following 6 days. In this case, there was a marked increase in the disease score and several of the mice injected with the anti-CTLA-4 died. Also, there was a considerable increase in the production of IL-2, TNF-α, and IFN-γ; EAE is usually accompanied by an increase in the production of these cytokines.[289]

Similar conclusions were drawn from another study.[290] In addition to the increase in the severity of the disease, histological studies showed more inflammatory foci in the brain and the spinal cord of the mice treated with the anti-CTLA-4 compared to the control antibody-treated mice. The authors concluded that blocking the inhibitory signal by CTLA-4 possibly resulted in enhanced activation and proliferation of antigen-reactive

T cells. The authors also stated that the exact mechanism of this improved activation is unclear and suggested that it was due to the lowering of the activation threshold of the T cells. The data from these studies showed that anti-CTLA-4 interfered with the CTLA-4/B7 interaction and prevented the inhibitory signal from being properly delivered, which led to enhanced T-cell activation and, consequently, more severe clinical disease. Both the studies suggest that CTLA-4 blockade is not a good approach for the treatment of autoimmune diseases, but they showed the importance of the CTLA-4/B7 interaction in these autoimmune diseases.

CTLA-4 blockade may show promise in the treatment of cancer. Immunosurveillance is a process in which the body protects itself from developing tumors. Unfortunately, in some individuals, the activation of the tumor-killing T cells is suppressed and, therefore, lowers the effectiveness of immunosurveillance.[291] It is believed that if the inhibitory signal sent by CTLA-4 is blocked by the anti-CTLA-4 antibody, the T cells can be activated and fight the tumors. Recently, several clinical trials have been conducted using the anti-CTLA-4 antibody to try to treat melanoma and other types of malignancies (Table 2). Two of these antibodies, ipilimumab and tremelimumab, have reached phase III clinical trials.[292]

1.4 Bifunctional peptide inhibitors

Recently, we have discovered a novel and selective method to suppress autoimmune diseases using bifunctional peptide inhibitor (BPI) molecules that simultaneously target both Signal-1 and Signal-2 (Fig. 4). Results from our studies have demonstrated that two different BPI molecules (i.e., PLP-BPI and GAD-BPI) can induce immunotolerance in

autoimmune diseases in two different animal models, EAE[293-294] and NOD mice.[161] In addition to these two BPI molecules, we also have ongoing and promising studies toward developing similar molecules to treat collagen-induced arthritis in DBA/1J mice, a mouse model for rheumatoid arthritis. In the case of PLP-BPI, the antigenic-peptide epitope derived from the proteolipid protein ($PLP_{139–151}$) was conjugated to LABL peptide derived from α_L integrin ($CD11a_{237–246}$) to make BPI molecules.[293-294] GAD-BPI is made from $GAD_{208-217}$ and LABL peptides. We hypothesize that the antigenic peptide fragment (i.e., $PLP_{139-151}$ and $GAD_{208-217}$) binds to MHC-II and the LFA-1 peptide fragment (LABL) binds to ICAM-1 on the surface of APC. Because both peptides are conjugated via a linker, simultaneous binding of BPI to MHC-II and ICAM-1 will prevent the translocation between TCR:MHC-II-peptide (Signal-1) and ICAM-1/LFA-1 complexes (Signal-2) that forms the immunological synapse. Inhibition of immunological synapse formation selectively alters the activation of T cells from T_H1 to T_H2 phenotypes and/or induces the production of T_{reg} cells. Because these BPI molecules contain specific antigenic peptides, we hope that we can target only a specific subpopulation of T cells involved in the onset and progression of autoimmune diseases without affecting the general immune response.

Studies with experimental models of autoimmune disease and allergy have shown that the administration of soluble peptides based on known T cell epitopes leads to suppression of the specific response, induction of bystander suppression, and prevention and treatment of hypersensitivity. Injections of antigenic peptides such as $PLP_{139-151}$[269, 295] and $GAD_{208-217}$[296] in saline have been shown to suppress the progression of EAE and

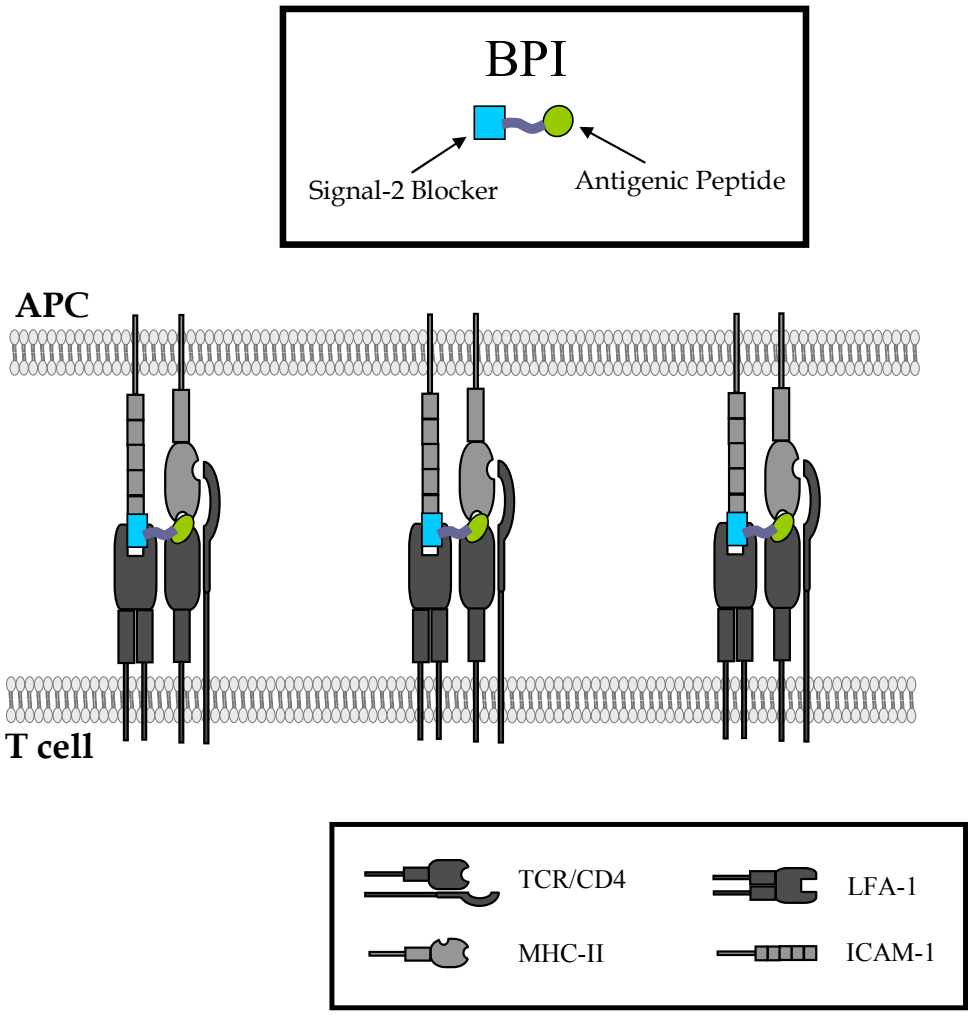

Figure 4. The proposed binding of the BPI molecule to MHC-II and ICAM-1, which inhibits the translocation of Signal-1 and Signal-2.

T1D, respectively, in mice models. However, treatment with antigenic peptides is potentially dangerous and can lead to fatal anaphylactic reactions. Many researchers have modified these antigenic peptides via mutation[297] or development of altered peptide ligands (APL)[298] in an effort to improve their therapeutic potential in treating autoimmune diseases as well as address their safety concerns.

Our data showed that PLP-BPI has better activity in suppressing EAE than do other peptides, including VP2-BPI (a BPI with an epitope peptide derived from Theiler's encephalomyelitis virus capsid protein, $VP2_{74-86}$), which is known to bind to MHC-II in SJL/J mice,[299] PLP-BPI_{sLABL} (PLP-BPI with a scrambled sequence of LABL), OVA-BPI (BPI in which $PLP_{139-151}$ has been replaced with $OVA_{326-337}$ derived from ovalbumin), and the unlinked mixture of $PLP_{139-151}$ and LABL. PLP-BPI-treated mice had very low EAE clinical scores and minimal loss in body weight compared to other groups. In addition, some of the PLP-BPI-treated mice did not develop the disease.[293-294] Similarly, dosing regimens designed for therapeutic treatment instead of prophylactic treatment showed that BPI was able to reverse disease severity very quickly.[294] Some of our unpublished work shows that PLP-BPI_{sPLP} (PLP-BPI with a scrambled sequence of $PLP_{139-151}$) has no EAE-suppressing activity, suggesting the significance of a unique structure in PLP-BPI such as the need for both $PLP_{139-151}$ and LABL peptides and covalent linking of these two peptides in the same molecule. Also, more importantly, we found out that these molecules are highly antigen-specific. We observed that splenocytes isolated from $PLP_{139-151}$-immunized mice responded to *in vitro* re-stimulation with $PLP_{139-151}$ but not MBP_{87-89} and vice versa.[294] It was also interesting to find that

splenocytes isolated from Ac-PLP-BPI-NH_2 (a modified form of PLP-BPI) showed significantly less proliferation in re-call to $PLP_{139-151}$, suggesting that injection of Ac-PLP-BPI-NH_2 reduces and/or suppresses the number of $PLP_{139-151}$-responsive populations. In a parallel study, GAD-BPI had the capacity to suppress the progression of T1D in NOD mice as demonstrated by significantly less insulitis and lower blood glucose levels in GAD-BPI-treated mice compared to control.[161]

Another important issue we looked at is the safety of these BPI molecules. As with any other peptide-based therapy, there is an associated risk involved in the use of BPI such as the possibility of an anaphylactic response, a life-threatening immediate hypersensitivity reaction caused by injections of the antigen-related peptides. Due to hypersensitivity reactions in patients during phase II clinical trials, development of an MS-targeted peptide drug was suspended.[300-301] In our studies, intravenous injection of PLP-BPI at four-to-five weeks post-immunization caused anaphylactic reaction to a much lower number of mice than does $PLP_{139-151}$. Additionally, a modified form of PLP-BPI (i.e., Ac-PLP-BPI-NH_2-2 with a longer linker and its N- and C-terminal acetylated and amidated, respectively) was found to be even less aggressive in inducing anaphylactic reactions.[294] We speculate that the lower incidence of anaphylaxis in BPI is due to the presence of LABL peptide that inhibits LFA-1/ICAM-1 interactions at the site of antigen recognition or the addition of another moiety at the N- or C-terminal to the parental allergic peptide $PLP_{139-151}$. Involvement of LFA-1/ICAM-1-mediated heterotypic aggregation of activated T cells to mast cells has been implicated in augmenting mast cell degranulation and histamine release.[302]

1.5 Peptide Safety

A potential problem that can arise when treating diseases with multiple injections of antigen-related peptides is the possibility of anaphylactic shock.[297, 303] Due to the fact that peptides are smaller in size than proteins, it is thought that they are safer and less likely to induce an anaphylactic response in the immune system. Despite the numerous advantages of peptides over proteins, anaphylactic reactions during treatments involving peptides have been widely described in the literature.[293, 297, 303]

In general, the mechanism of anaphylactic response is thought to occur when an antigen crosslinks with IgE bound to FcεRI on mast cells, which leads to degranulation and the release of histamine and cytokines, both of which are inflammatory mediators.[302] The administration of anti-IgE antibodies along with myelin-specific peptides during the treatment of EAE has been shown to inhibit anaphylaxis, which suggests that IgE plays a large role in anaphylaxis when treating with peptides.[304]

APL have been shown to suppress anaphylaxis in EAE without affecting its activity toward myelin-reactive T cells.[305] Short linear peptide sequences generally lack the ability to crosslink adjacent IgE molecules on mast cells and basophils.[306] Because a non-immunogenic peptide sequence can become immunogenic when only one of its amino acid residues is altered, care must be exercised in designing an APL so that it retains its activity toward its target and does not become more antigenic.

The avoidance of IgE activation is necessary to circumvent allergic response. It is optimal to use peptides shorter than 20 amino acids, as longer peptides have been associated with more adverse events compared to their shorter counterparts.[306] Five-to-six

amino acids have been shown to be sufficient minimal antigenic determinants.[307] Alternatively, APL that can bypass the body's ability to create an anaphylactic response against a peptide without affecting its activity toward its target have been shown to work and must be utilized in creating effective therapies.

1.6 Mechanism of peptides that treat autoimmune diseases

It has been described before that the immune system functions as a balance between pathogenic effector cells and regulatory cells.[306] The suppression and activation of these two will lead to either an immunogenic response or an immunotolerant response (Fig. 5). Autoimmune diseases typically arise due to an imbalance between these two responses. Therefore, to combat autoimmune diseases, we must try to restore balance in the immune system. One way to restore the balance is by either inducing the suppressive cells or activating the regulatory cells.

DCs are considered to be the most prominent APCs in inducing an autoimmune disease. They are believed to send a "danger" signal to induce an immunogenic response, but that is not always the case.[308] The function of the DC depends on whether it is in a mature (activated) or immature state.[309] The maturation of DC into a T cell stimulatory mode is activated by an inflammatory stimulus that is initiated by the uptake of an insoluble antigen (Fig. 5, left side).[306, 309] Once the DC is in the mature state, its phenotype changes and there is an upregulation of co-stimulatory and adhesion molecule expression on its cell surface. Presentation of antigen in this way leads to the differentiation of proinflammatory cellular responses.[306] Such an inflammatory cellular

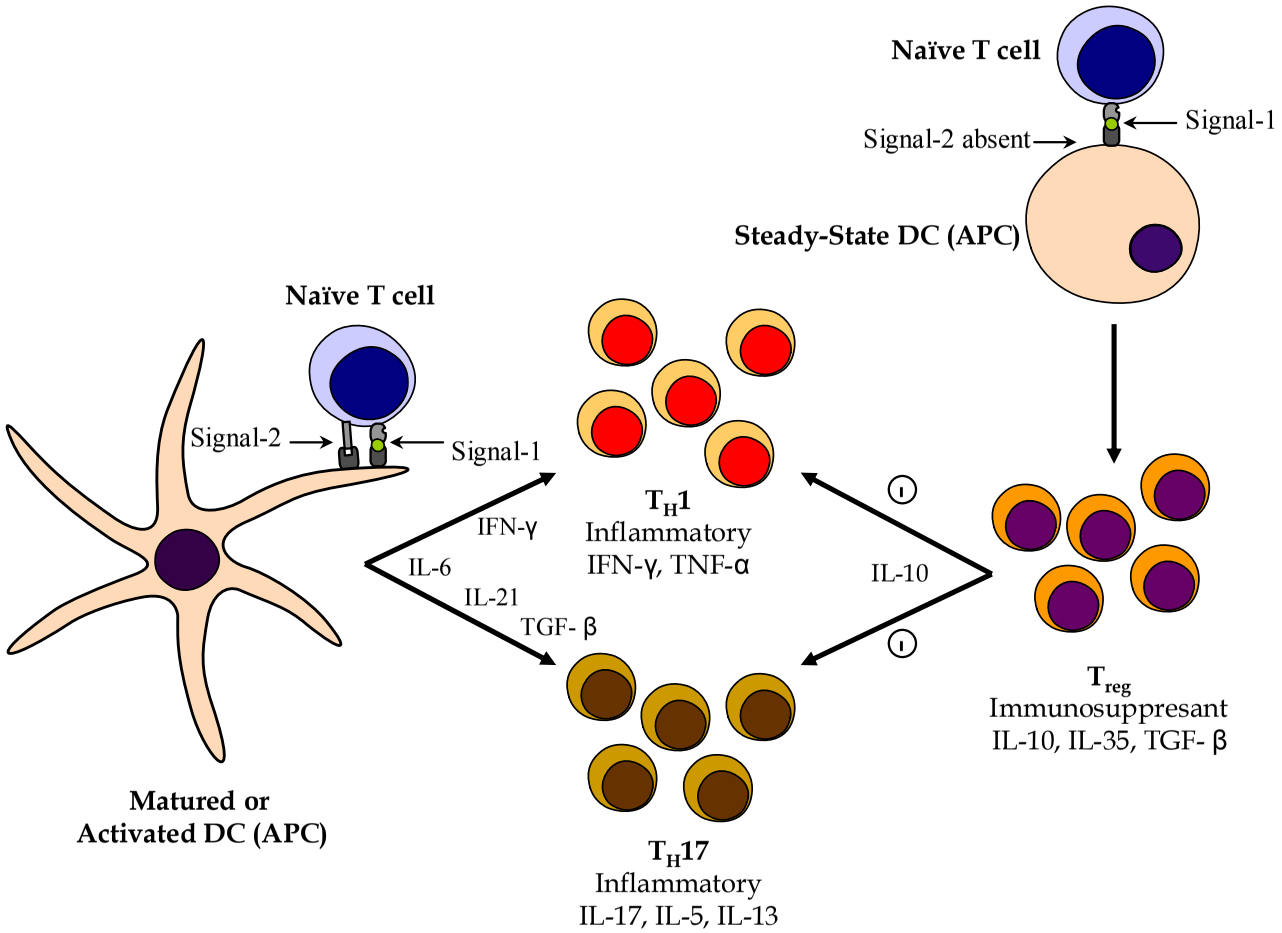

Figure 5. The proposed mechanism of activation and suppression of immune response during autoimmune diseases. On the left, interaction between mature/activated DC and naïve T cells is mediated by MHC-II antigen/TCR complex (Signal-1) in the presence of the co-stimulatory signal (Signal-2) that leads to differentiation into T_H1 and T_H17 cells. On the right, interaction between immature/steady-state DC and naïve T cells is mediated by MHC-II antigen/TCR complex (Signal-1) in the absence of the co-stimulatory signal (Signal-2) that leads to differentiation into T_{reg} cells. T_{reg} cells produce IL-10 that suppresses the proliferation of T_H1 and T_H17.

response triggered by a mature DC (mDC) includes the induction of T_H1 cells that produce IFN-γ and IL-2.[309] Other proinflammatory responses include the induction of T_H17 and the release of IL-17.[135] These inflammatory responses triggered towards self tissues usually lead to an autoimmune disease such as multiple sclerosis.

On the other hand, an immature DC (iDC) is one that did not take up and process any antigens. It has been shown, using antibodies specific for peptide-free MHC-II molecules that iDC express empty MHC-II molecules, on their surface and can bind antigenic peptides in solution. These bound antigens can be presented to T cells without internalization and processing.[310] Soluble peptides that are presented by iDC lead to a differentiation of T_{reg} cells (Fig. 5, right side).[306] Activation of regulatory T cells leads to the production of regulatory cytokines such as IL-10 and TGF-β. Such cytokines have been shown to have an important role in ameliorating autoimmune diseases in animal experimental models.[311-313] In the case of some autoimmune diseases, expanding the "regulatory pool" leads to a downregulation of the effector response (T_H1) and enhancement of the immunosuppressive response (T_H2).[306]

1.7 Proposed BPI mechanisms

There are several possible mechanisms that could explain how BPI molecules suppress the activation of T cells and result in suppression of autoimmune diseases. The first of these is that only the antigenic fragment of BPI molecule binds to MHC-II on the iDC (steady-state DC) and is presented to naïve T cells for differentiation to produce T_{reg} cells that generate IL-10 (Fig. 6, right side). This proposed mechanism, which is similar to the

mechanism of action of soluble antigenic peptide as a therapeutic vaccine,[306] ignores the role of cell adhesion peptide (e.g., cIBR or LABL) on the BPI molecule. It has been shown that injection of the BPI molecules generates IL-10[293]; however, the kinetics and the amount of IL-10 production upon BPI and PLP peptide injections have not been compared. Because the BPI molecule has better efficacy than the parent antigenic peptide (PLP),[293] it is predicted that the amount of IL-10 produced by injecting BPI molecules will be higher than that produced by injecting PLP peptide. The second possible mechanism is that the BPI molecule binds not only to MHC-II but also to adhesion molecules (e.g. LFA-1 or ICAM-1) on iDC. Thus, during the interaction between BPI-loaded iDC and naïve T cells, the BPI molecule blocks the immunological synapse formation to induce differentiation of naïve T cells to T_{reg} cells. The presence of cell adhesion peptide on BPI makes it a more efficient modulator for naïve T cell differentiation than the antigenic peptide alone. The third possible mechanism is that the BPI molecule simultaneously binds to MHC-II and LFA-1 to block the immunological synapse formation during the interaction between naïve T cells and mDC (Fig. 6, left side) to suppress the differentiation of naïve T cells to T_H1 and T_H17 cells. There is some indication that the BPI molecule induces the generation of IL-4-producing T_H2 cells, which may tip the balance to lower the production of T_H17 and T_H1 cells. The increase in IL-4 production was observed after BPI injection into the EAE mouse model.[293] The mechanism of action of BPI molecules may also be a combination of these three proposed mechanisms.

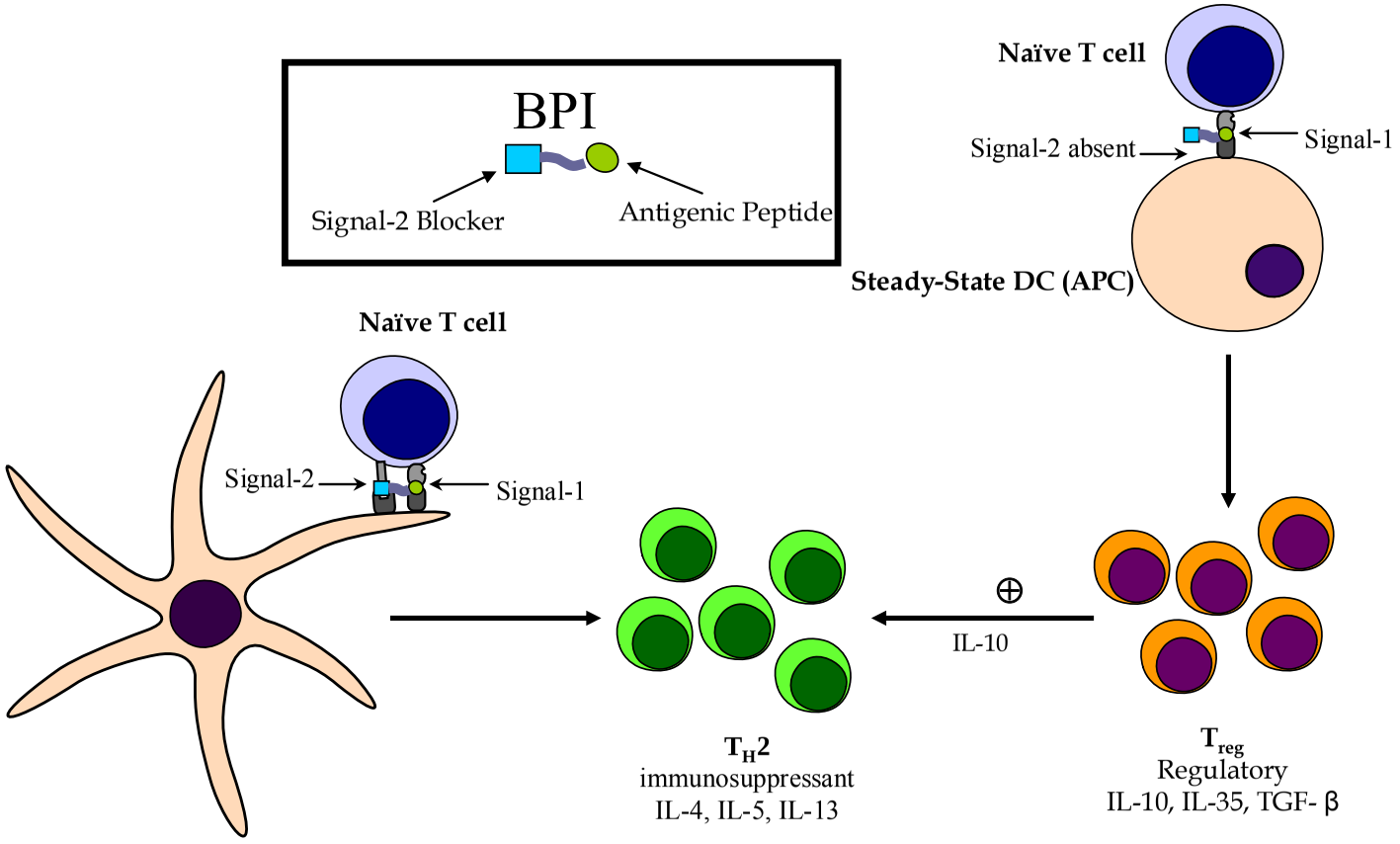

Figure 6. Potential mechanism of BPI molecules in altering the differentiation of naïve T cells upon interaction with immature and mature DC. On the right, interaction of steady-state/immature DC with BPI molecule produces differentiation of naïve T cells to IL-10-producing Treg cells. IL-10 induces the proliferation of immunosuppressant T_H2 cells. On the left, interaction of BPI molecule with mature/activated DC induces immunosuppressant T_H2 cell differentiation.

1.8 Conclusions

Immunomodulating compounds have been explored as potential treatments for autoimmune diseases such as MS, T1D, and RA among others. However, most of the agents developed thus far aim at broad modulation of the immune response and, therefore, may present some undesirable side effects. Targeted drug delivery is a more attractive strategy to improve the efficacy and reduce the side effects of these immunomodulating drugs. In our studies, we hope to design bifunctional peptide inhibitors that target only a subpopulation of T cells responsible for the progression of the disease without affecting the general immune response. Although it has not been clearly elucidated, BPI-based therapies work either by inhibiting the formation of the immunological synapse by blocking both Signal-1 and Signal-2 or shifting the T cell subpopulation into T_{regs} and/or T_H2-like phenotype. The detailed mechanisms of action of BPI-type molecules, including the antigen-specific immunosuppressive activity as well as its safety, are being investigated in depth in our current studies. Additionally, we are looking at ways of improving the structure and sequence of BPI to provide a more efficient and well-tolerated immunotherapy.

1.9 Objectives and specific aims

One way to improve the structure of the BPI molecule is by substituting the cell adhesion peptide with a cell adhesion protein. For example, the original PLP-BPI molecule contains an antigenic-peptide epitope, $PLP_{139–151}$, and a cell adhesion peptide, LABL. The LABL peptide derived from the I-domain of α_L integrin ($CD11a_{237–246}$) is

known to bind to ICAM-1 receptors. Thus, LABL can be replaced with the I-domain, to make a conjugate between I-domain and PLP (PLP-I-domain). The hope is that the PLP-I-domain will have improved efficacy, safety, and tolerability. In addition, I-domain can also be used to deliver multiple antigenic peptides. Hence, the overall objective of this work is to investigate the utility of the I-domain protein in delivering antigenic peptides to antigen presenting cells for suppressing EAE in mouse model. Therefore, the following specific aims were pursued:

Specific Aim 1: To conjugate a fluorescent dye (FITC) to the N-terminus and/or lysine residues of the I-domain protein to produce FITC-labeled I-domain. To characterize the conjugate and identify the FITC-modification sites in the FITC-I-domain protein using LC ESI-Q-TOF mass spectrometry.

Specific Aim 2: To examine the binding and internalization properties of the FITC-I-domain protein, mediated by ICAM-1 receptors on the surface of lymphocytes.

Specific Aim 3: To conjugate PLP to the N-terminus and/or lysine residues of the I-domain protein to produce PLP-I-domain and evaluate its *in vivo* suppression of EAE.

The above specific aims are addressed in chapter 2, 3, and 4 respectively.

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CHAPTER 2

Rapid Identification of Fluorochrome Modification Sites in Proteins by LC ESI-Q-TOF Mass Spectrometry

2.1 INTRODUCTION

Proteins (including antibodies) are modified with fluorochromes to maximize sensitivity in binding assays or cell imaging. Fluorescein and rhodamine are the most commonly used fluorochromes because of their high quantum yields. The modification of proteins by either fluorescein-5′-isothiocyanate (FITC) or rhodamine-B-isothiocyanate (RITC) is a well-established method.[1] Protein labeling is done by reacting the isothiocyanate group of the fluorescent dye with the primary amines and thiol groups of proteins to yield thiourea or dithiourethane adducts.[2] Frequently, the reaction product between a protein and an activated dye is a heterogeneous mixture of conjugates that differ in the number of fluorochromes attached to the protein. Identification of modified sites on the protein is often a laborious process and is challenging because of the heterogeneity of the sample and low abundance of the modified residues. However, it can be critical to determine the modified sites, as the affinity of the protein to its ligand may be altered by modification of critical residues in the binding site or alteration of the protein conformation.

Lymphocyte function-associated antigen-1 (LFA-1) belongs to an integrin receptor family that is composed of heterodimeric α- and β-subunits. The α-subunit of LFA-1 has an inserted domain (I-domain), which serves as the primary binding domain for intercellular adhesion molecule-1 (ICAM-1). The I-domain protein has been expressed and is well characterized. The metal ion-dependent adhesion site (MIDAS) of this I-domain interacts with a glutamate residue of domain 1 (D1) of ICAM-1.[3-4, 5] It has been shown that the cell surface expression of ICAM-1 is upregulated during several

pathological conditions, including cancers, inflammation, and autoimmune diseases (multiple sclerosis,[6-8] rheumatoid arthritis,[9] type 1 diabetes,[10] and lupus[11]). Thus, our goal is to utilize the I-domain to target small molecule drugs to cells with upregulated expression of ICAM-1 protein for lowering side effects.

In this study, the I-domain was conjugated with fluorescein and rhodamine fluorophores at lysine residues and the N-terminus (Fig. 1). The I-domain has 20 lysine residues that can be modified by fluorophores, and the condition for the conjugation reaction was optimized to introduce an average of three to four fluorescein molecules per I-domain molecule or two to three rhodamine molecules per I-domain molecule. It is essential to determine the sites of modification on the I-domain because the modification may influence the binding and internalization properties of I-domain. Modification of the MIDAS region could render the protein inactive or unable to bind to ICAM-1.

A combination of tryptic digest and mass spectrometry was used to identify the conjugation sites of the fluorophores because it is a sensitive and flexible method for the characterization of modified peptides. The fluorescein thiocarbamoyl modification has been detected by mass spectrometry since the early use of soft ionization, thermospray, or chemical ionization to detect phenyl thiohydantoin (PTH) derivatives of amino acids, the result of phenyl isothiocyanate (PTC) coupling to amines.[12] While the focus of this early work was differentiating between the amino acids in the classic Edman degradation, the spectra also presented a “reporter ion” of the modifying group. The reporter ion is readily generated in an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) instrument such that the modified peptides can be found and their identity can be

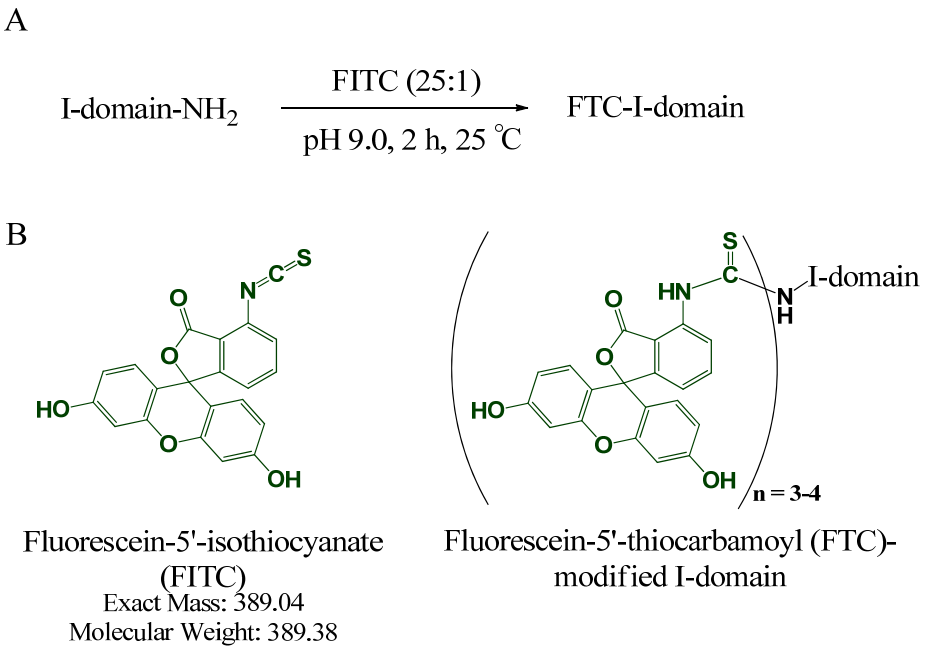

Figure 1. **(A)** Schematic representation of a single-step modification of I-domain protein with FITC. **(B)** Illustration of chemical structures of FITC and FTC-I-domain.

confirmed. This strategy of using fragmentation in the collision cell of a Q-TOF without explicit MS/MS has been exploited in the fatty acid analysis of complex lipids analysis[13] and provides the fundamental data used in MSE by WATERS for proteomics.[14] Previously, Schnaible and Przybylski demonstrated the possibility of the identification of fluorescent dye modified sites by increasing the declustering potential (so called "cone" fragmentation) in the electrospray ion (ESI) source to generate a reporter ion from the dye on a peptide.[15] This specific fragmentation was utilized for assigning the fluorescent dye modification sites in peptides obtained from enzymatic digestion of fluorescein-modified hen egg white lysozyme (HEL). In the present study, we used ESI-Q-TOF MS to readily generate these reporter ions in the collision cell of the Q-TOF and, along with LC/MS, expand the earlier observations of Schnaible and Przybylski to assess the fluorochrome distribution and determine the fluorescent dye conjugation sites in the modified I-domain after trypsin digestion.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Fluorescein-5′-isothiocyanate (FITC) isomer I, rhodamine-B-isothiocyanate (RITC) mixed isomers, and other solvents and reagents of highest available purity were purchased from Sigma-Aldrich (St. Louis, MO). Sequence-grade modified trypsin was purchased from Promega (Madison, WI).

2.2.2 Peptide synthesis and purification

The model peptides cyclo(1,12)Pen-ITDGEATDSGC (cLABL) and cyclo(1,12)Pen-PRGGSVLVTGC (cIBR) were synthesized as linear peptides by standard Fmoc solid-phase peptide chemistry using the automated peptide synthesis system (Pioneer™ PerSpective Biosystems, Framingham, MA) as described elsewhere.[16] The synthesis product was purified by HPLC followed by cyclization and purification as previously described.[16] The molecular weight of the peptide was confirmed by electrospray ionization mass spectrometry (M + 1 = 1174.5 for cIBR and 1197.25 for cLABL).

2.2.3 Fluorochrome modification of the peptides

Conjugation of FITC with cLABL was carried out using the procedure previously published by our laboratory.[17] Briefly, pure peptide (0.04 mmol) was dissolved in 5 mL f Nanopure water and FITC (0.08 mmol) was added. The pH of the solution was adjusted to 10 by addition of a 1.0 N NaOH solution. The solution was stirred for 1 h with a magnetic stirrer. After completion of the reaction time, the reaction mixture was neutralized by the addition of a 10% v/v acetic acid solution. The solution was lyophilized. The lyophilized crude peptide was then purified by semi-preparative C_{18} reversed-phase HPLC. The pure fractions of the peptide were pooled and lyophilized. The lyophilized FITC-peptide was analyzed by analytical C_{18} reversed-phase HPLC for its purity, and its identity was confirmed by ESI-MS (M+1). Conjugation of rhodamine with cIBR was done in a manner identical to that described above with the exception that FITC was replaced by RITC.

2.2.4 LFA-1 I-domain protein expression and fluorochrome modification

The LFA-1 I-domain protein (residues 128–307, plus an additional N-terminal methionine) was expressed, refolded, and purified as previously described.[18] Briefly, the expression vector pET-11d containing the gene of LFA-1 I-domain$_{128\text{-}307}$ was used in *E. coli* BL21 (Stratagene, La Jolla, CA) competent cells, where the protein was found in inclusion bodies. The protein was purified and refolded as previously described.[18] The identity was confirmed by mass spectrometry.

The procedure for conjugation of fluorescent dyes (FITC/RITC) with the I-domain protein was adopted from the previously published method with some modifications.[1] To a total of 6.0 mg of the I-domain in PBS containing 10 mM $MgSO_4$, a one-fourth volume of 1.0 M $NaHCO_3$-Na_2CO_3 buffer at pH 9.0 was added. FITC (5 mg/mL in DMSO) was added to the protein solution to a 25-fold molar excess over the I-domain with a final concentration of 1.5 mg/mL. The reaction was carried out for 2 h at 25 °C in the dark with constant stirring. The reaction was quenched with addition of 0.1 N HCl. Separation of the free dye from dye-coupled protein was done using a Superdex 200 size-exclusion column. The fractions belonging to the conjugated protein were collected and concentrated using an ultrafiltration device. The concentrated protein was filtered using a 0.2-micron filter and stored at 4 °C. Modification of I-domain with rhodamine was done in a manner identical to that described above with the exception that a 25-fold molar excess FITC was replaced by a 20-fold molar excess of RITC. The number of FITC or RITC groups conjugated to the I-domain protein was determined by electrospray mass spectrometry.

2.2.5 In-gel tryptic digestion and LC ESI-Q-TOF mass spectrometry analysis

100 μg of protein was run on a polyacrylamide gel followed by Coomassie blue staining. The stained gel spot of interest was excised and cut into smaller pieces for better solvent penetration into the gel. Then, the gel pieces were transferred to a clean microcentrifuge tube, washed twice with a solution containing a 1:1 mixture of acetonitrile and 50 mM ammonium bicarbonate buffer at pH 8.0 for 45 min at 37 °C with gentle agitation. After discarding the washing solution, the gel pieces were shrunk with 250 μL of neat acetonitrile. After 10–15 min the residual solvent was discarded, and the gel pieces were dried completely. The gel pieces were re-swelled with 40 μL of digestion buffer A (0.2 M ammonium bicarbonate buffer at pH 8 and 5 mM $CaCl_2$) containing 2 μg trypsin. Then, 50 μL of the digestion buffer B (0.2 M ammonium bicarbonate buffer at pH 8 and 10% acetonitrile) was added to keep the gel pieces immersed throughout the digestion. The digestion was carried out overnight at 37 °C, and the reaction was stopped by the addition of 0.1% formic acid. The gel pieces were sonicated and the supernatant containing the digested peptides was collected as primary sample fraction and analyzed by LC ESI-Q-TOF MS.

All HPLC separations were performed with a Water Acquity solvent delivery system using a binary gradient of solvent A composed of 98.92:1:0.08 H_2O/formic acid/TFA (vol/vol/vol) and solvent B containing 98.92:1:0.08 acetonitrile/H_2O/formic acid (vol/vol/vol). The primary sample fraction (20 μL) containing the digested peptides was separated on a C_4 (5 cm × 1 mm i.d.) reversed-phase HPLC column (VM5C4W, packed by Micro-Tech Scientific, Vista, CA) with a linear gradient from 10% to 70% B in 60

min followed by a wash and re-equilibration step. Further, the HPLC system was coupled online to the electrospray source of a Q-TOF-2 mass spectrometer (Micromass UK Ltd., Manchester, UK). Mass spectra were acquired with instrument cone voltage 35 eV, collision energy 10 eV with Ar in the collision cell. The instrument was set up such that ESI-MS spectra were acquired in positive reflector mode with a scan time of 6 s and in the mass range of 200-3000. The instrument was calibrated using NaI.

2.2.6 Analysis of the peptide fragments

The reporter ion chromatograms were processed for molecular weight of the peptides using MaxEnt3 in the MassLynx V4.1 software (Micromass UK Ltd.) to reduce the spectral complexity to single charge representation. A theoretical list of all the possible peptides as a result of tryptic digestion of the protein and their corresponding masses was generated by BioLynx. Each peptide fragment peak identified in the chromatograph was manually compared with peptide mass from the theoretical list.

2.3 RESULTS

2.3.1 Analysis of I-domain conjugates by ESI-MS:

The I-domain protein was modified with fluorescent dye (FITC or RITC) in an aqueous buffer solution using a molar ratio of fluorescent dye:I-domain of 25:1 or 20:1 for 2 hrs at 25 °C at pH 9.0 as shown in Fig. 1. Under such conditions, the isothiocyanate group of the fluorescent dye preferentially reacts with primary amino and thiol groups on proteins forming stable thiourea and dithiourethane adducts. Because the I-domain does

not contain any cysteine residues, it is expected that only a thiourea adduct is formed. After removal of the unreacted fluorescent dye by SEC, the number of conjugated FITC molecules was ascertained using ESI-Q-TOF MS. The deconvoluted spectrum shows seven prominent peaks (Fig. 2). Each adjacent peak is separated by a mass difference of 388 Da to 390 Da with a mean of 389 Da. This consistent mass difference between the adjacent peaks in the spectra is due to the covalent link of the FITC group (molecular weight 389.4 Da) on the I-domain protein. The mass of the first peak in the series is 21,072 Da (1F, one fluorescein), which is associated with I-domain conjugated with one FITC molecule. The parent I-domain protein has a molecular weight of 20,682 Da (0F, no fluorescein). In this case, the associated error is less than 0.01%, which is typical for such mass measurements. The rest of the peaks can be assigned to the I-domain conjugated with two, three, four, five, six, and seven covalently linked FITC (2–7F) molecules, respectively. The spectrum also shows the disappearance of the peak for the parent I-domain (0F), indicating a complete conversion. A similar analysis done using RTC-I-domain shows 0–4 covalently linked RITC molecules (data not shown).

2.3.2 Analysis of model peptide conjugates by ESI-MS:

Before analyzing the tryptic-digest products of I-domain conjugates, the fragmentation profile of peptide-conjugated with fluorescent dye was first investigated using Q-TOF mode. cLABL peptide was reacted with FITC at the N-terminus to give FTC-cLABL,

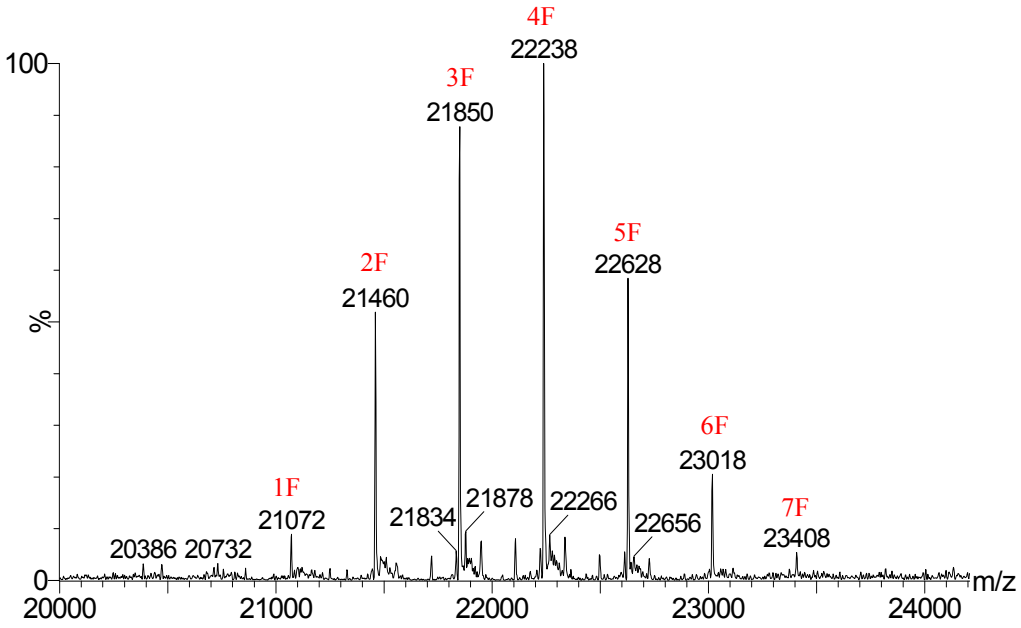

Figure 2. Deconvoluted mass spectrum of FTC-I-domain. The modification of one FITC molecule to either lysine or N-terminus amino group increases the theoretical mass of the I-domain protein by 389 Da. F, is the number of FITC molecules conjugated.

and cIBR peptide was reacted with RITC to produce RTC-cIBR. After the HPLC purification, the pure FTC-cLABL or RTC-cIBR was used for ESI-MS analysis.

Under the conditions employed, the presence of the reporter ion (i.e., free dye molecule) was detected. In this case, several fragments were detected, including the free dye (i.e., FTC, m/z = 389.98 or RTC = m/z of 500.26), the parent peptide, and the modified peptide. Free fluorescent dye molecules were facile at lower energy and were readily generated. By following the reporter ion, the peptide fragments attached to the fluorescent dye can be identified. These results suggest the possibility of identifying the conjugation sites in I-domain protein by tracing the modified and unmodified peptide fragments.

2.3.3 Determination of conjugation sites in the I-domain:

For determination of FITC conjugation sites, a classical peptide-mapping technique involving trypsin digestion and ESI-Q-TOF mass spectrometry followed by computer-facilitated data analysis of peptides was performed. It is well known that trypsin cleaves the C-terminal Lys and Arg residues; however, the modified Lys residues in the I-domain cannot be clipped by trypsin. Thus, the total ion chromatograms (TIC) from the tryptic digest of FTC-I-domain and parent I-domain proteins were compared (Fig. 3A-B). The TIC shows differences in the profiles, peak intensities, and retention times. For example, the increase in the retention time of the dye-modified peptides is due to the increase in hydrophobicity of the peptide fragment conjugate to the dye. Because of the large differences between the chromatograms of tryptic digests of FTC-I-domain and I-domain,

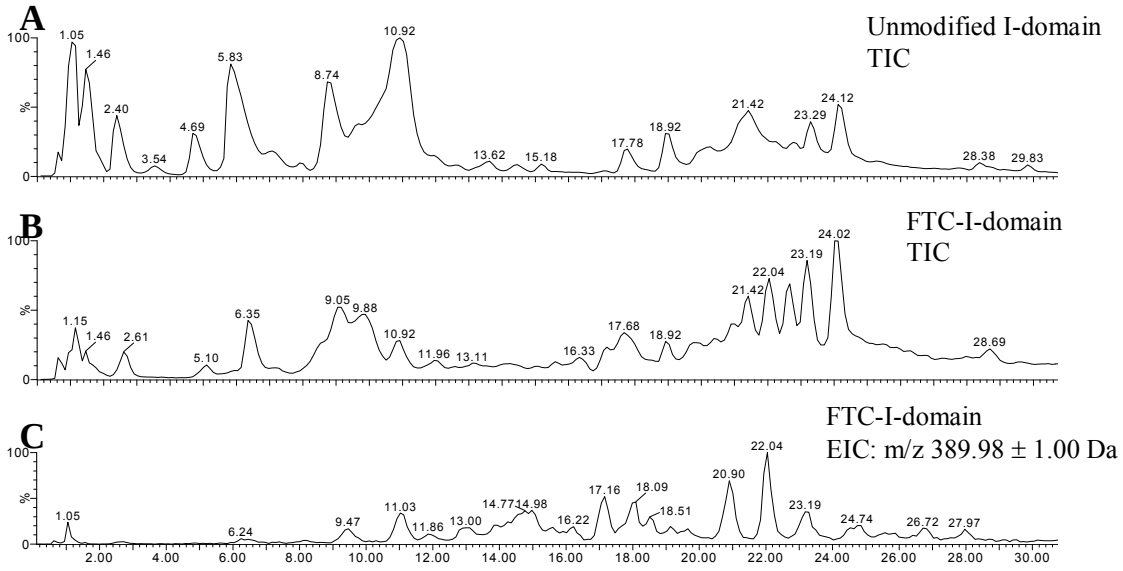

Figure 3. The ion chromatograms of tryptic digests of unmodified I-domain and FTC-I-domain. **(A)** The total ion chromatogram (TIC) of unmodified I-domain. **(B)** The TIC of FTC-I-domain. **(C)** The extracted ion chromatogram (EIC) of FTC-I-domain. The EIC was obtained by separating all the peaks containing the reporter ion (FITC; 389.98 Da ± 1.00 Da) from the TIC.

the identification of the conjugation sites using UV and/or fluorescence traces was laborious and time-consuming. Therefore, MS data were used to identify the modified peptides. During the analysis, the assumptions were that the modifications occurred at lysine residues and the expected mass of the uncleaved tryptic peptides increased by 390 Da for FTC-modified peptides and 500 Da for RTC-modified peptides. Interestingly, MS data of the dye-modified proteins showed a unique peak that could be assigned to the mass of the free fluorescent dye molecule (reporter ion with a mass of 389.98 Da or 500.26 Da), similar to that found in the model peptide analysis. Thus, the observed free dye molecule was derived from a specific peptide fragment upon cleaving the dye moiety (i.e., FTC or RTC) during collision-induced dissociation (CID).

The facile release of reporter ion was fundamental for the data analysis. Considering this key observation, the extracted ion chromatograms (EIC) containing the reporter ion FITC (389.98 Da) or RITC (500.26 Da) were pulled out of the total ion current (chromatograms) in the LC/MS profile of the modified I-domain. A similar extraction was also done from unmodified protein profile (data not shown). The EIC of the FTC-modified protein shows several peaks that appeared between 7.2 and 30.0 min (Fig. 3C), indicating that the amino group of the lysine side chain and/or the N-terminus of methionine could be modified in these peptides. Then, each new peak from the EIC of the FTC-I-domain in the ESI-MS spectra was processed using MaxEnt3 deconvolution algorithm. The processed spectra were then searched for three ions: reporter ion (FTC), FTC-modified peptide, and its corresponding unmodified peptide.

The ESI-MS spectrum of a peak eluting at 22.04 min from the tryptic-digest of FTC-I-domain is shown in Fig. 4A. The deconvoluted spectrum shows three prominent ions (Fig. 4B), including m/z 389.98 Da, which corresponded to the FTC moiety, m/z 2056.69 Da, which correlated with the unmodified peptide (^{151}FAS…FE^{168}K; Cal: 2057.10 Da), and m/z 2445.66 Da, which corresponded to the modified FTC-peptide T18-19 (^{151}FAS…^{154}K…FE^{168}K or ^{151}FAS…^{161}K…FE^{168}K). The FTC-peptide T18-19 has modification either at ^{154}K or ^{161}K. In addition, the ESI-MS spectrum of another peak eluting at 27.97 min shows a fourth ion with m/z 2834.55 Da, which corresponds to an FTC-peptide with modification at both the ^{154}K and ^{161}K residues. However, there are populations of protein that were not modified at both ^{154}K and ^{161}K residues. This is due to the observation of peptides T18 (m/z 1210.42) and T19 (m/z 865.31) derived from complete trypsin cleavage.

In summary, a total of 8 and 6 modification sites were identified in the FTC-I-domain and RTC-I-domain, respectively. Tables 1 and 2 show a complete list of modified peptides obtained from the FTC-I-domain and RTC-I-domain. In all the identified peptides, the conjugation sites were assigned to the internal lysine residue except for peptide T1, where the conjugation site was assigned to the primary amino group or the N-terminus of the first amino acid residue, methionine. Interestingly, most of the conjugation sites found in the RTC-I-domain were similar to those found in the FTC-I-domain.

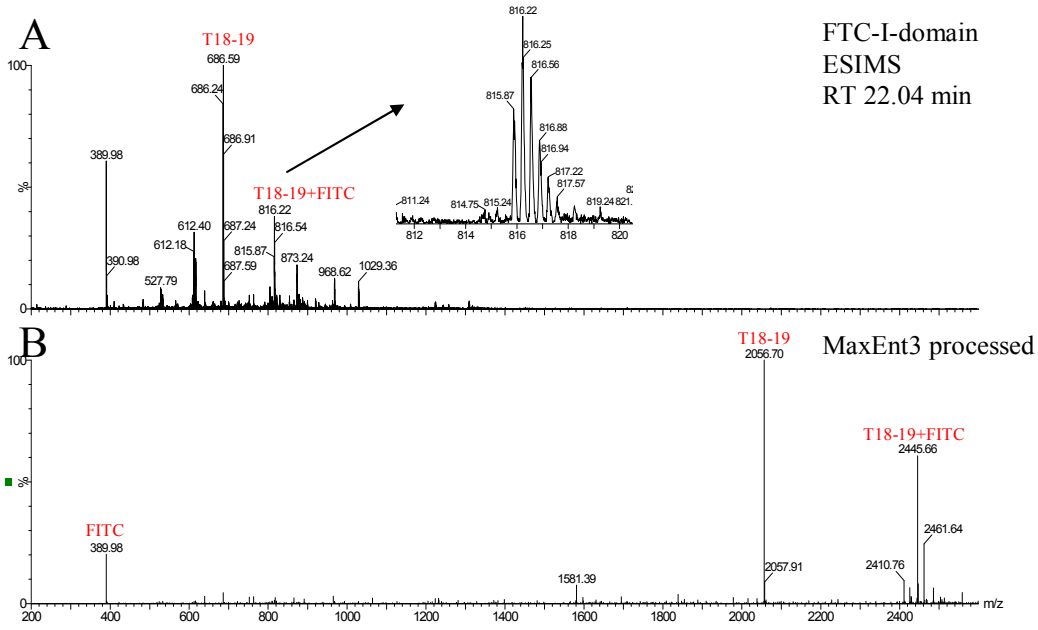

Figure 4. The ESI-MS and MaxEnt3 processed spectra of the peak eluting at 22.04 min derived from the LC/MS analysis of the tryptic digests of FTC-I-domain. **(A)** The ESI-MS of the peak eluting at 22.04 min derived from the LC/MS analysis of the tryptic digests of FTC-I-domain, inset shows the spectrum of FITC modified T18-19 peptide. **(B)** The MaxEnt3 processed spectrum of peak eluting at 22.04 min derived from the ESIMS spectra of the tryptic digests of FTC-I-domain.

Table 1. FITC modification sites in the FTC-I-domain detected in peptides from trypsin digestion		
Modified peptide	Sequence	Modified sites
T1	^{1}M^{23}K	1M
T2-3	^{24}ILDFMKDVM^{33}K	29K
T4-5	^{34}KLSNTSYQFAAVQFSTSY^{52}K	34K
T6-7	^{53}TEFDFSDYVK^{63}R	62K
T18-19	^{151}FASKPASEFVKILDTFE^{168}K	154K, 161K
T20-21	^{169}LKDLFTELQ^{178}K	170K
T21-22	^{171}DLFTELQK^{179}K	178K

Table 2. RTC modification sites in the RTC-I-domain detected in peptides from trypsin digestion		
Modified peptide	Sequence	Modified sites
T1	^{1}MGNVDLVFLFDGSMSLQPDEFQ^{23}K	1M
T2-3	^{24}ILDFMKDVM^{33}K	29K
T4-5	^{34}KLSNTSYQFAAVQFSTSY^{52}K	34K
T15-16	^{131}YIIGIGKHFQT^{142}K	137K
T18-19	^{151}FASKPASEFVKILDTFE^{168}K	154K, 161K

2.4 DISCUSSION

The conjugation method adopted for the preparation of the I-domain-fluorescent dye conjugates yields samples with a heterogeneous distribution of fluorochrome groups covalently linked to the lysine residues. However, the reaction conditions have been optimized to maintain batch-to-batch reproducibility. Each batch consistently showed the same number of fluorochrome modifications per molecule of protein as determined by whole protein MS analysis and UV. Also, as a quality control tool, CD spectrometry results showed reproducibility of the spectra of modified proteins.

The FTC-I-domain preparation contained one to seven linked FTC molecules (Fig. 2), while the RTC-I-domain preparation contained one to four linked RTC molecules (data not shown). Out of 21 modifiable residues (20 lysine + N-terminus methionine), there are eight sites modified by FTC and six sites modified by RTC (Tables 1 and 2). In addition, all of the identified sites were partially modified. There are possibly low abundant modification sites that cannot be detected during LC/MS analysis. The MS data analysis using the reporter ion can identify almost all of the modified peptides in relatively less time. Also, such analysis can aid in identifying low-abundance modified peptides that fall within the detectable range of the instrument. However, very low levels of modified peptides that fall below the detection range of the instrument may not be detected; as a result, we cannot deny the presence of more than the observed number of modification sites. This study shows a combination of tryptic digest and LC ESI-MS analysis is proven to be a powerful technique in identifying the modification sites.

Identification of sites of FTC- or RTC-modification of the I-domain is necessary because the modified I-domain serves two main purposes. The first purpose is that the labeling of I-domain increases the sensitivity of detection of the protein during *in vitro* binding studies. It is hoped that conjugation with the fluorescein and rhodamine does not alter the structure and binding site of the I-domain. Secondly, characterization of the modification sites provides an idea regarding the reactivity and availability of certain lysine residues for conjugation with drug molecules within the I-domain. Neither of these goals may be accomplished if the residues at the MIDAS region and the structure of I-domain have been altered to make the molecule inactive.

Our results indicate that FTC-moieties are found at lysine residues away from the MIDAS region (Fig. 5) and that the FTC-I-domain binds to ICAM-1 on Raji cells (Chapter 3). The binding properties of FTC-I-domain to ICAM-1 on the cell surface are concentration and temperature dependent. Recently, the I-domain has been conjugated to antigenic peptides for targeting to antigen-presenting cells (APC) for controlling T-cell differentiation in autoimmune diseases (Chapter 4). The antigen-I-domain conjugate can suppress the progress of experimental autoimmune encephalomyelitis (EAE) in the mouse model, a model for multiple sclerosis. Therefore, it is necessary to study the mechanisms of binding the I-domain protein to intercellular adhesion molecule-1 (ICAM-1 or CD54) proteins on the surface of leukocytes. Thus, the I-domain of LFA-1 protein was modified with fluorochrome, and cellular binding properties were characterized using human leukocyte cell lines (i.e., Raji cells).

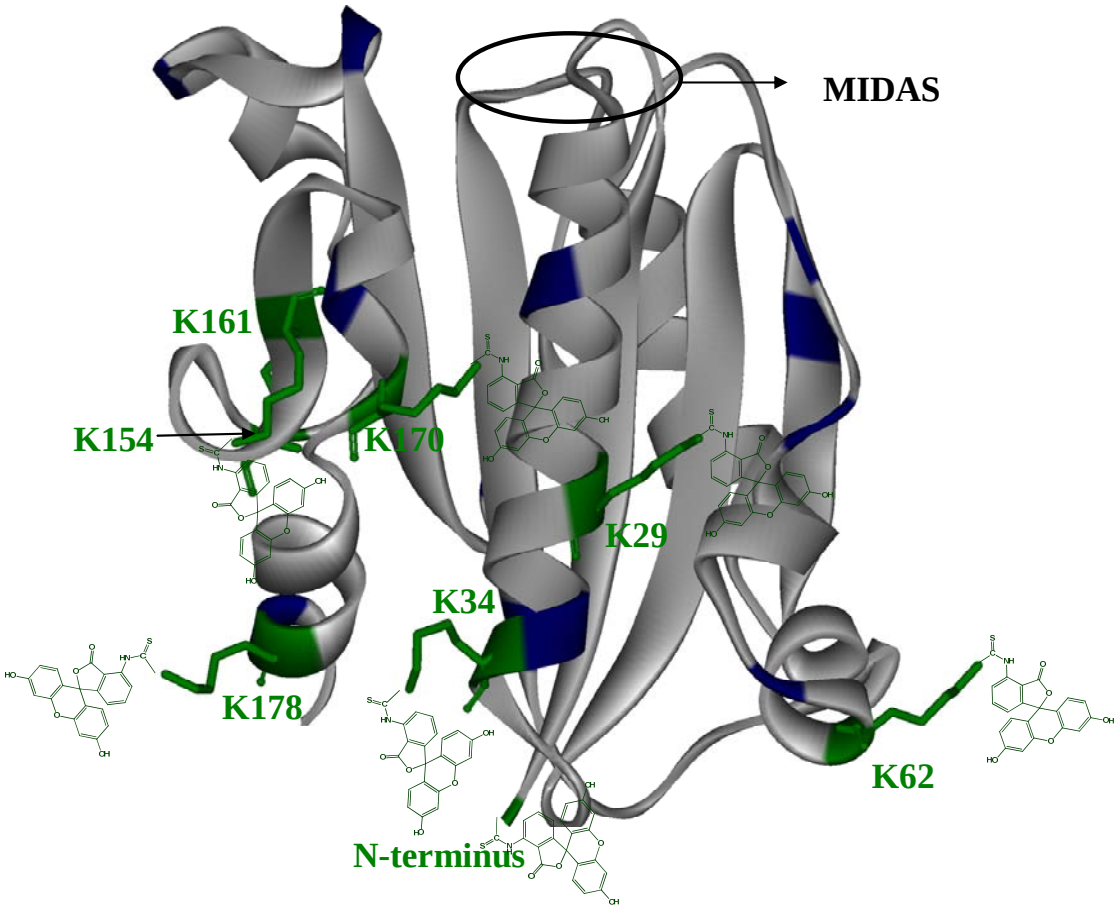

Figure 5. The X-ray structure of I-domain (PDB code 1ZON). The residues in green are the FITC modification sites and the residues in blue are the unmodified lysine modification sites. The modified lysine residues and the N-terminus are labeled. The protein images were created using Accelrys DS Visualizer 1.7.

2.5 CONCLUSIONS

The reporter ion quickly allowed identification of peaks modified with peptides in a complex peptide chromatogram. The observation of reporter ion and peptide represent the neutral loss; can be extended to ion trap instruments that lack low mass detecting capabilities.

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CHAPTER 3

Characterization of I-domain Binding Properties to Leukocytes

3.1 INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1) is an Ig-like transmembrane glycoprotein constitutively expressed on several cell types, including leukocytes and dendritic cells. ICAM-1 is an attractive target for drug delivery to immune cells, since its expression is elevated in several autoimmune disorders.[1-7] Increased ICAM-1 expression has been observed on T-cells isolated from synovial fluid and brain tissue of patients suffering from rheumatoid arthritis and multiple sclerosis, respectively.[1-3, 8-9] Elevated ICAM-1 expression is thought to be a direct consequence of inflammatory cytokines released upon the infiltration of T-cells at the site of destruction. The infiltration of T-cells progressively modifies the clinical outcome of the autoimmune diseases by enhancing the immunogenic response. Cell adhesion molecules (CAM), including ICAM-1, are internalized into the cell cytoplasmic domain via a unique pathway referred to as CAM-mediated endocytosis.[10-13] Ligands that bind to CAM can be used to selectively target drugs for intracellular delivery to immune cells expressing these upregulated adhesion molecules. Therefore, I-domain can be utilized to target drugs to cells with upregulated ICAM-1 during inflammation. ICAM-1 targeting offers not only surface binding but also intracellular drug delivery.

The natural counter-receptor of ICAM-1 is leukocyte function associated antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18), a transmembrane cell surface glycoprotein.[14-15] It belongs to the integrin superfamily of adhesion molecules, widely expressed on immune cell subsets. It is comprised of heterodimeric α (180kDa) and β (95kDa) subunits, which are non-covalently associated.[16-17] The α-subunit (CD11a) of LFA-1 consists of an amino

terminal-inserted-domain (I-domain, approximately 200 amino acids), which is sufficient for LFA-1 binding to ICAM-1. The I-domain is structurally located at the top of the α-subunit with a central five-stranded parallel β-sheet surrounded by seven α-helices; it has two important sites for modulation of binding to ICAM-1.[17-18] The first site is a unique metal ion-dependent adhesion site (MIDAS). The I-domain binds to domain-1 (D1) of ICAM-1 through its MIDAS, which involves coordination of a divalent cation (i.e., Mg^{2+} or Ca^{2+}) via the Asp137, Ser139, Ser141, Thr206 and Asp239 residues.[19] The second important site is the I-domain allosteric site (IDAS); IDAS is an important binding site for small molecule inhibitors of ICAM-1/LFA-1-mediated cell-cell adhesion. Although the I-domain protein has long been studied, its potential use as a drug targeting molecule has not been investigated to date. Therefore, we began to investigate the utility of the I-domain to deliver drugs and antigen peptides to immune cells for controlling autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type-1 diabetes.

In this work, we studied the binding and uptake properties of the fluorescence-labeled I-domain (FITC-I-domain) by ICAM-1 on Raji cells to evaluate the possible applicability of the I-domain to target drugs to cells with upregulated ICAM-1 for improving drug efficacy and lowering side effects. ICAM-1 was chosen because it can undergo endocytosis and cellular recycling.[10-13] In this case, several of the lysine residues of the I-domain were derivatized with fluorescein isothiocyanate (FITC) to generate FITC-I-domain. The binding properties of FITC-I-domain to ICAM-1 on Raji cells after incubation at 4 °C and 37 °C were characterized by flow cytometry as well as binding modulation using anti-I-domain and anti-ICAM-1 antibodies. The cellular localization of

FITC-I-domain was determined by confocal microscope, and the amounts of the I-domain on the cell surface and in the intracellular compartments were determined using fluorescence intensity integrations. Finally, the effect of divalent cations on binding FITC-I-domain to ICAM-1 was also determined.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Fluorescein isothiocyanate (FITC) isomer-I, phorbol 12-myristate-13-acetate (PMA), tumor necrosis factor-α (TNF-α) and human IgG were purchased from Sigma-Aldrich (St. Louis, MO). 4',6-Diamidino-2-phenylindole (DAPI) and Alexa Fluor® 647 phalloidin were purchased from Molecular Probes (Eugene, OR). Anti-human-ICAM-1 mAb to domain D1 (clone 15.2), anti-human-ICAM-1 to domain D2 (clone 8.4A6), mouse IgG1 (clone MOPC 31C), anti-human LFA-1 CD11a (clone 38), mouse IgG2a (clone RPC 5), and polyclonal goat anti-mouse IgG/IgM FITC were purchased from Ancell Cooperation (Bayport, MN). Anti-human-ICAM-1 mAb to domain D1 (clone RR1/1) was purchased from Millipore (Billerica, MA).

3.2.2 Cell culture

Cells were purchased from ATCC (Rockville, MD and propagated in an RPMI-1640 medium containing 10% v/v fetal bovine serum, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 2.0 g/l $NaHCO_3$. The cells were maintained at a density

of 1.5×10^6 to 2×10^6/mL at 37 °C in 95% humidified and 5% CO_2 atmosphere. As necessary, MOLT-3 cells were activated in medium containing PMA for 16 h or TNF-α for 24 h with final concentrations of 0.2 μM and 10 ng/mL, respectively.

3.2.3 Protein expression and purification

The expression of I-domain has been described elsewhere.[20] Briefly, the I-domain DNA sequence was subcloned into pET-11d vector followed by transformation into competent *E. coli* BL21 cells (Stratagene, La Jolla, CA). Then, the *E. coli* cells were cultured and the induction of protein overexpression was done using isopropyl D-thio-galactoside (IPTG, Sigma-Aldrich). For isolating the protein, the cell pellets were lysed in 10 mL of homogenization buffer (HB) using a French press followed by centrifugation (20000 × g) at 4 °C for 1 h. The majority of the I-domain was found in the protein pellet. After washing, the cell pellet was resuspended in 15 mL of denaturing buffer (DB, 6 M guanidine-HCl and 50 mM Tris, pH 8.5) and incubated for 1 h at room temperature before centrifugation for 30 min to remove the remaining cell debris. The supernatant was diluted to a protein concentration of 1 mg/mL and concentrated with Amicon® ultrafiltration cell (Millipore) with 5,000 MWCO ultra-filtration membranes. After dialysis against RFB and PBS containing 10 mM $MgSO_4$ at 4 °C, the folded I-domain protein was purified by passing it through a Superdex 200 size-exclusion column (Amersham Biosciences, Pittsburgh, PA).[21] The protein was concentrated to 10 mg/mL, and the concentration was measured at 280 nm using an extinction coefficient of 8940 M^{-1} cm^{-1}. The purity of the I-domain was determined by SDS-PAGE gel and confirmed by

electrospray ionization mass spectrometry (ESI-MS) with an m/z ratio of 1881.2 corresponding to [M + 11H]. The secondary structure of the folded protein was determined using far-UV circular dichroism (CD).

3.2.4 Conjugation of FITC to the I-domain to give FITC-I-domain

The conjugation of FITC to the I-domain protein was performed by following the method previously described.[22] Briefly, one-fourth volume of 1.0 M $NaHCO_3/Na_2CO_3$ buffer pH 9.0 was added to the I-domain solution (6 mg/mL) followed by addition of a 25-fold molar excess of freshly prepared FITC solution (5 mg/mL) in DMSO. The mixture was stirred in the dark for 2 h at 25 °C. At the end of the reaction, the pH was readjusted to 7.4 using 0.1 N HCl. Immediately the mixture was purified to separate the conjugated protein from free FITC using a Superdex 200 size-exclusion column. The fractions for the FITC-I-domain were collected and concentrated by ultrafiltration. The concentration of the pure conjugated protein was determined using a UV method described previously[22] by measuring the absorbance at 280 nm and 495 nm following the equation:

$$\text{Concentration of I-domain protein (mg/mL)} = [A_{280}-(0.35 \times A_{495})]/0.432$$

where 0.432 is the A_{280} of I-domain at a concentration of 1.0 mg/mL and $0.35 \times A_{495}$ is the correction factor due to the absorbance of FITC at 280 nm. The purity of the FITC-I-domain conjugate was confirmed by SDS-PAGE gel, and the number of FITC molecules conjugated to the I-domain protein was determined by ESI-MS. The effect of conjugation

on the secondary structure was evaluated by comparing the CD spectrum of the I-domain conjugate and that of the parent I-domain.

3.2.5 Flow cytometry

3.2.5.1 *Cell preparation:* The cell preparation described here was carried out in the same manner for all the experiments described below. Cells from the stock were centrifuged at 500 × g for 5 min and then resuspended in sterile PBS to reach a concentration of 5×10^5/mL. Aliquots of 1.0 mL of the cell suspension were added to 1.5 mL centrifuge tubes followed by centrifugation at 500 × g. The resulting supernatants were carefully aspirated without disturbing the cell pellets; the pellets were used for the following experiments.

3.2.5.2 *Determination of cell surface expression of ICAM-1 receptor:* To decrease the non-specific binding, 20 µL of human IgG (300 µg/mL) in FACS buffer containing PBS (10 mM sodium phosphate, 150 mM sodium chloride pH 7.2-7.5, 1% BSA, and 0.05% sodium azide) was added to the cell pellets and incubated for 5 min at 4 °C. Then, 80 µL of anti-CD54 (clone 15.2) or isotype control primary antibody sub-stock ranging from 20 to 0.0006 µg/mL dilutions in FACS buffer was added to the cells, followed by incubation for 45 min at 4 °C. The cells were washed twice with 0.5 mL FACS buffer and centrifuged at 500 × g for 5 min. Into the cell pellet, 50 µL of FITC-labeled secondary antibody (1:60 dilution) was added and incubated for 30 min at 4 °C, followed by extensive washing with FACS buffer. The cell pellet was resuspended in 300 µL 2%

paraformaldehyde/PBS and stored at 4 °C prior to analysis. The samples were analyzed using a FACScan apparatus (Becton Dickinson, Franklin Lakes, NJ) equipped with CELL QUEST software program. As many as 10,000 cells were counted for every sample during acquisition, and each experiment was done at least in triplicate. Cells without antibody treatment were used as controls. The control histogram was placed within 10^0 to 10^1 on the log scale of fluorescence intensity by adjusting the fluorescence detector. The binding intensities were represented as their relative values to the reference conditions and were determined from the mean values of the histograms for cell number and log fluorescence intensity (mean fluorescence intensity (MFI) corrected for non-specific fluorescence).

3.2.5.3 *Concentration- and temperature-dependent binding of the FITC-I-domain protein:* The cell pellet was resuspended in PBS containing increasing concentrations of FITC-I-domain protein from 0 to 52 μM at 4 °C and up to 156 μM at 37 °C and incubated for 60 min. At the end of the incubation period, the cells were washed three times with FACS buffer, and the cell suspension was centrifuged at 500 × g for 5 min. The cells were fixed using 2% paraformaldehyde/PBS. The resulting samples were analyzed using flow cytometry, and the MFI was determined as described above.

3.2.5.4 *The effect of anti-ICAM-1 or anti-LFA-1 mAb on FITC-I-domain binding:* Binding of FITC-I-domain to ICAM-1 was also evaluated in the presence of anti-ICAM-1 and anti-LFA-1 (anti-CD11a) mAb. For anti-ICAM-1 mAb, the cells were first treated

with 20 µl human IgG (300 µg/mL in FACS buffer) for 5 min at 37 °C followed by pre-incubation for 30 min at 37 °C with 30 µl of anti-ICAM-1 (either clone 15.2, RR1/1 and 8.4A6) or isotype controls mAb with the final mAb concentration of 0.2 µM. Then, the cells were incubated with 50 µl of FITC-I-domain (24 µM) for another 1 h at 37 °C. The cell suspension was centrifuged, washed, and fixed before flow cytometry analysis.

For anti-LFA-1 mAb blocking, 5 µM FITC-I-domain was first incubated with equimolar amounts of anti-LFA-1 (clone 38) mAb or its isotype control antibody in buffer for 30 min at 37 °C; then, this mixture was added to cells pretreated with human IgG and incubated for 1 h at 37 °C. The cell suspension was centrifuged, washed, and fixed before analysis by flow cytometry.

3.2.5.5 *Divalent cation-dependent binding of the FITC-I-domain protein:* The cells were incubated for 1 h at 37 °C with FITC-I-domain protein (25 µM) in the absence and presence of 1.5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $CaCl_2$/EDTA, $MgCl_2$/EDTA, or $MnCl_2$/EDTA in PBS. After incubation, the cells were treated as shown above prior to flow cytometry analysis.

3.2.5.6 *Time- and temperature-dependent binding of the FITC-I-domain protein:* The cells were incubated with 100 µL of 25 µM FITC-I-domain prepared in FACS buffer for 0, 5, 10, 15, 30, 60, 120, 180, and 360 min at 4 °C and 37 °C. After washing and fixing, the cells were subjected to flow cytometry analysis.

3.2.6 Confocal microscopy study

Raji cells were centrifuged (500 × g) for 5 min and re-suspended in sterile warm PBS to a final concentration of 2.5 × 10^5/mL, and 1.0 mL aliquots were dispensed into nine centrifuge tubes. After centrifugation (500 × g) and removal of the supernatant solution, 10 μL of FACS buffer was added to cells pellets, and the cell suspension was equilibrated for 5 min at either 4 °C or 37 °C. Then, 15 μL of FITC-I-domain (100 μM) was added to the cell suspension and incubated for 0, 5, 10, 15, 30, 60, 120, 180, and 360 min. At each time point, cells were centrifuged, washed twice (with FACS buffer), and fixed with 100 μL of 4% paraformaldehyde in PBS. To the fixed cells, 100 μL of 16.5 nM Alexa Fluor® 647 phalloidin for actin staining and 12.5 μL of 5 μM DAPI for nuclear staining were added followed by overnight incubation at 4 °C. The following day, these samples were mounted on a slide and imaged using a Yokugawa CSU-10 spinning disk confocal unit attached to an Olympus IX-81 inverted microscope platform (Olympus America, Inc., Center Valley, PA) Images were captured using a Hamamatsu C9100 electron multiplier 1000 × 1000 pixel CCD camera and the SlideBook software package (Intelligent Imaging Innovations, Denver, CO). The resulting images were analyzed using the program CellProfiler[23] to segment regions of interest for measuring fluorescence intensity associated with the membrane and the cytoplasm for each cell examined.

3.2.7 Statistical analysis

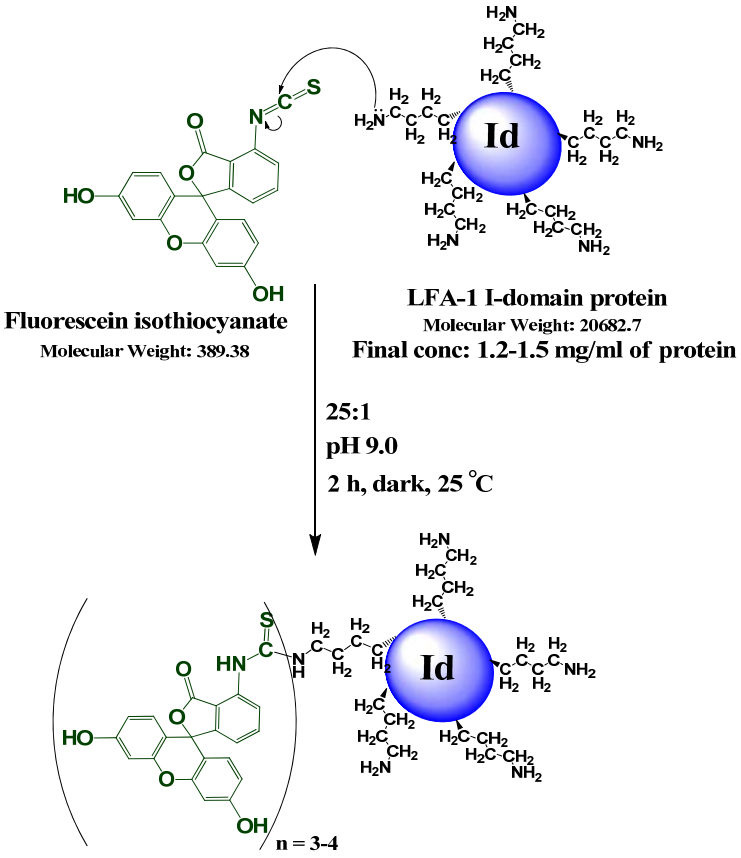
All the values obtained in the above experiments were expressed as mean ± SE. The differences between groups were tested for statistical significance using Student's t-test. The presence of significant difference is denoted with *p*-values of < 0.05 or < 0.01.

3.3 RESULTS

3.3.1 Fluorescein conjugation to I-domain:

The I-domain has been successfully conjugated with fluorescein group at several of the 20 lysine residues to make FITC-I-domain (Scheme 1). Reproducibility of the conjugation was achieved by optimizing the pH, ratio of I-domain/FITC, temperature, and reaction time. The FITC-I-domain was easily separated from the excess FITC using SEC (Fig. 1A). The pure FITC-I-domain shows only a single band on SDS-PAGE when stained with Coomassie blue or upon shining UV light on the gel (lane 4, Fig. 1B). In contrast, the crude reaction product shows two spots by Coomassie blue and three fluorescent spots by UV light (lane 2, Fig. 1B) and the parent I-domain only shows one spot by Coomassie blue and no spot found by UV (lane 3, Fig. 1B).

The ESI-MS data indicate one to seven FITC groups attached to the I-domain with the average of 3.5 FITC groups per I-domain molecule (top panel, Fig. 1C). There is no unconjugated I-domain with a MW of 20,682 found in the MS spectrum (bottom panel, Fig. 1C). The CD spectrum of FITC-I-domain is similar to the spectrum of unmodified I-domain (Fig. 1D) suggesting that there is no alteration in the secondary structure of



Scheme 1. Reaction of I-domain protein with FITC along with the reaction conditions listed.

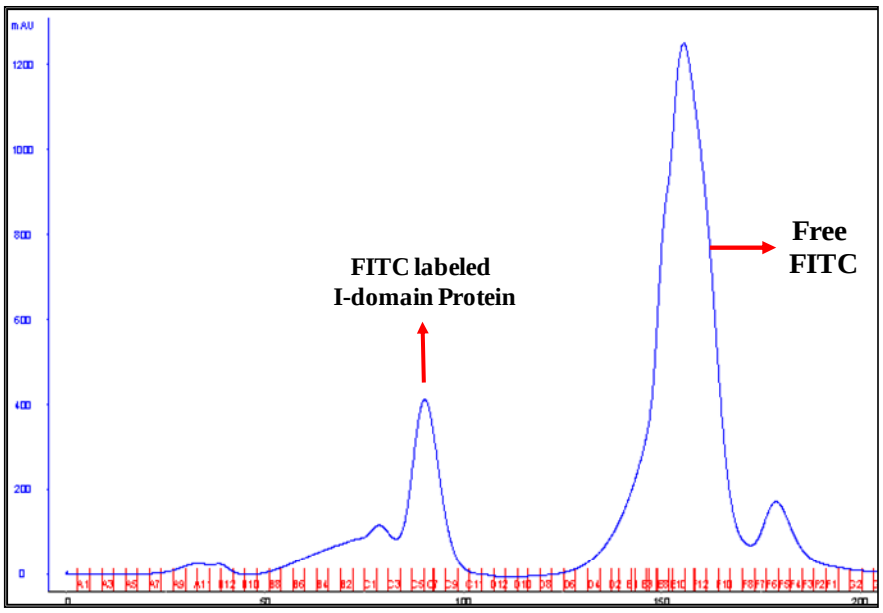


Figure 1A. Separation of the FITC-conjugated I-domain protein from the unreacted (free) FITC using size-exclusion chromatography (SEC).

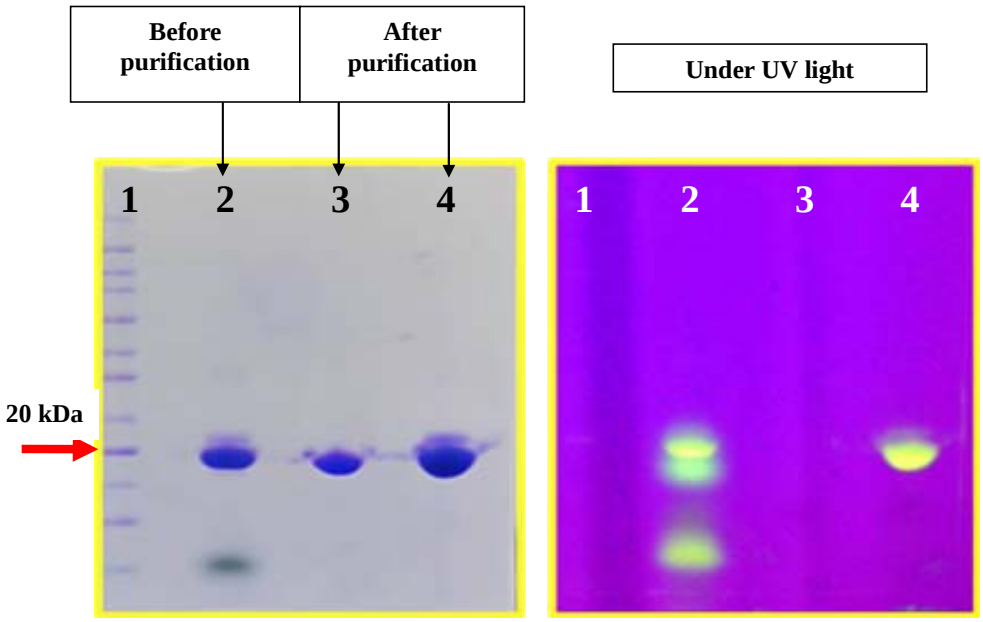


Figure 1B. SDS-PAGE analysis of pure FITC-I-domain protein after staining with Coomassie blue (left) and before staining under UV light (right): molecular weight marker (lane 1), reaction mixture of I-domain protein and FITC (lane 2), unmodified I-domain protein (lane 3), and FITC-conjugated I-domain protein (lane 4).

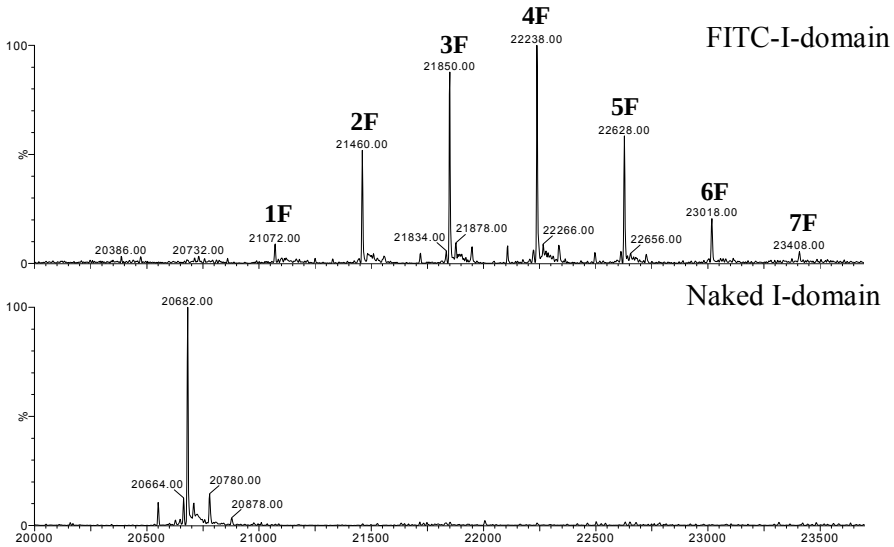


Figure 1C. ESI-MS analysis of the FITC-I-domain protein (top) and the I-domain protein (bottom).

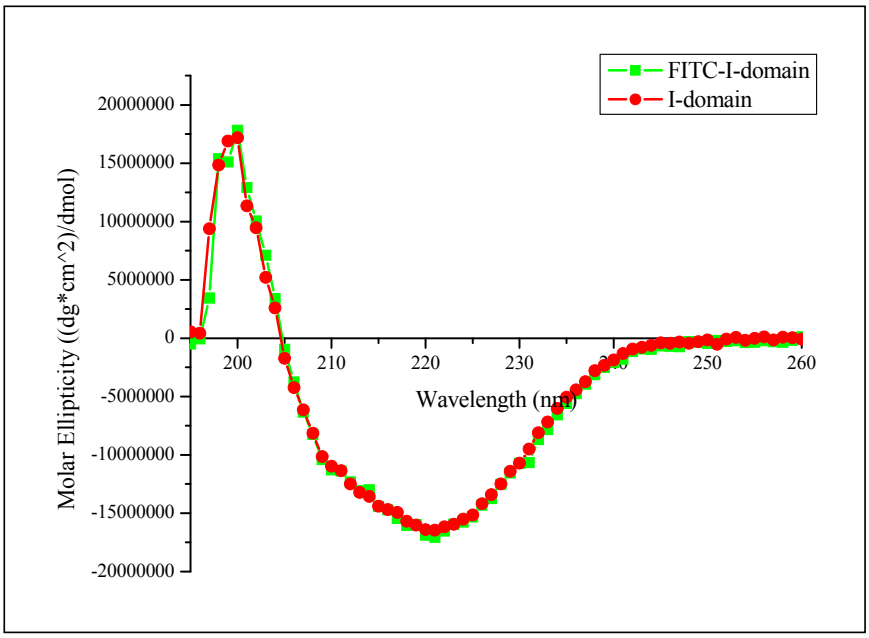


Figure 1D. CD spectra of unmodified I-domain protein (red), FITC conjugated I-domain protein (green).

FITC-I-domain. The predicted secondary structure of FITC-I-domain from CD spectra shows 37% α-helix and 26% β-sheet, which are similar to the secondary structure content from X-ray crystallography (i.e., 37% α-helix and 22% β-sheet).[24]

3.3.2 Comparison of ICAM-1 expression on Raji, HL-60, and Molt-3 cells:

The ICAM-1 expressions on HL-60, Molt-3 T-cells and Raji cells were determined using anti-ICAM-1 D1 (clone 15.2) mAb to select the appropriate cells for FITC-I-domain binding studies (Fig. 2). In all three cell lines, the anti-ICAM-1 mAb binding increased upon an increase in concentration, and the binding eventually saturated at higher concentrations. The highest ICAM-1 level was found in Raji cells followed by Molt-3 cells and HL-60 cells. As a negative control, the isotype mAb did not show any appreciable binding to all three cells. Upon induction with TNF-α or PMA, the expression of ICAM-1 was slightly increased in Molt-3; however, the increase in ICAM-1 expression did not match the amount of ICAM-1 on the un-activated Raji cells. Based on these results, the Raji cells were used to perform binding experiments using FITC-I-domain protein.

3.3.3 FITC-I-domain binding to ICAM-1 on Raji Cells:

At 4 °C, the FITC-I-domain exhibited an increase in binding with saturation around 30 μM of added protein (Fig. 3A). At 37 °C, there was no observable saturation even up to threefold higher concentration (0–156 μM, Fig. 3B). At 37 °C, the results suggest that

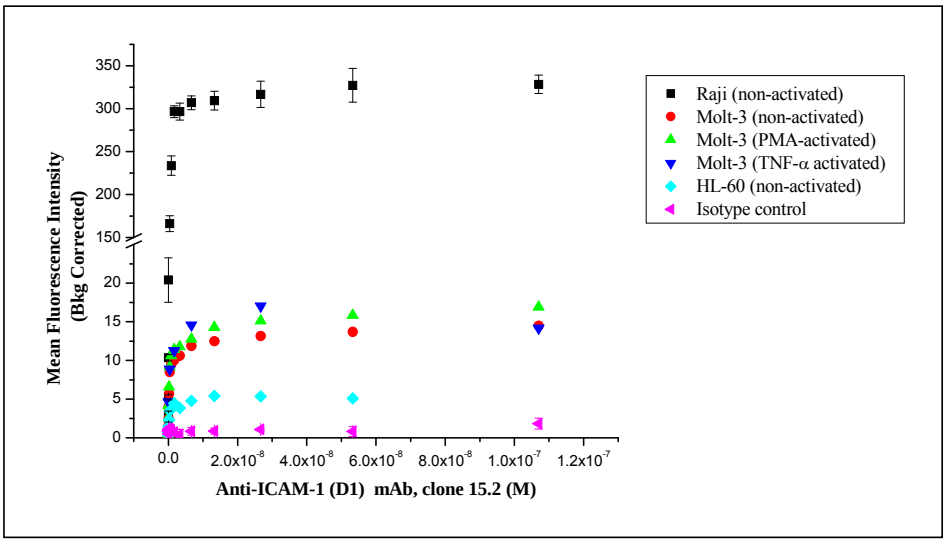


Figure 2. Comparison of the surface expression of ICAM-1 receptors in different cell lines using anti-ICAM-1 (CD54) mAb 15.2. Data shown from one representative experiment ± S.E. (n = 3) for Raji cells and (n = 1) for Molt-3 and HL-60.

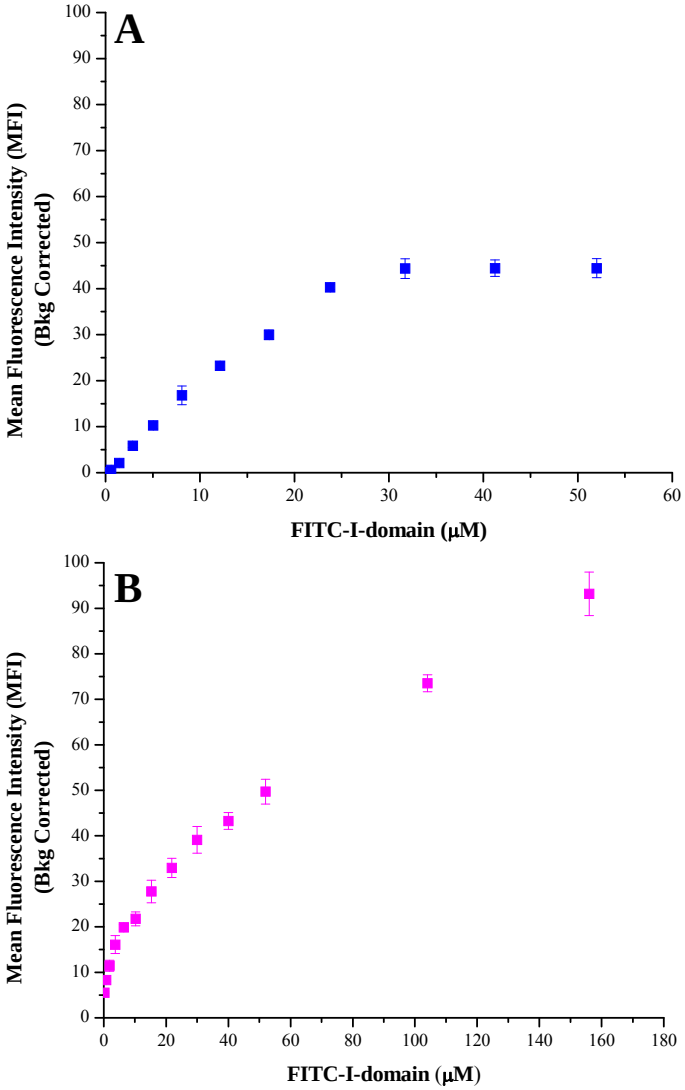


Figure 3. Binding of FITC-I-domain to ICAM-1-expressing Raji cells at 4 °C **(A)** and 37 °C **(B)**. The results are expressed as the mean ± S.E. (n = 3).

there is binding as well as receptor-mediated uptake of FITC-I-domain. Confocal microscopy was used to study the uptake properties of the conjugate.

3.3.4 The effect of anti-ICAM-1 and anti-LFA-1 mAb on FITC-I-domain binding:

The effect of anti-LFA-1 (anti-CD11a, clone 38) and anti-ICAM-1 mAb on binding of FITC-I-domain to ICAM-1 was determined to access its ICAM-1 specificity. Anti-CD11a mAb binds to I-domain of LFA-1. It blocked 55% of FITC-I-domain binding to Raji cells; in contrast, the isotype control mAb did not block the FITC-I-domain binding (Fig. 4A). The result from this experiment is consistent with previous results by others using cell based assay[25] and solid phase ELISA assay.[26]

Instead of blocking, all three anti-ICAM-1 mAb (clone RR1/1, 15.2, and 8.4A6) enhanced binding (164%, 146%, and 179%, respectively) of FITC-I-domain to ICAM-1 on Raji cells (Fig. 4B) compared to the isotype control mAb. For RR1/1 mAb, the obtained results were unexpected because this mAb recognizes the Gln-73 residue of domain-1 of ICAM-1[27] and it an effective blocker of ICAM-1/LFA-1-mediated cell-cell adhesion.[28] Anti-ICAM-1 clones 15.2 and 8.4A6 that recognize domain-1 and -2 of ICAM-1, respectively, are also inhibitors of ICAM-1 binding to LFA-1.

3.3.5 Ca^{2+} enhances binding of FITC-I-domain to ICAM-1:

The active state of I-domain interacts with Glu-34 of ICAM-1 via its MIDAS region through a coordination of a divalent metal ion. Thus, the effect of various divalent cations on binding affinity of FITC-I-domain to ICAM-1 on Raji cells was evaluated. Ca^{2+}

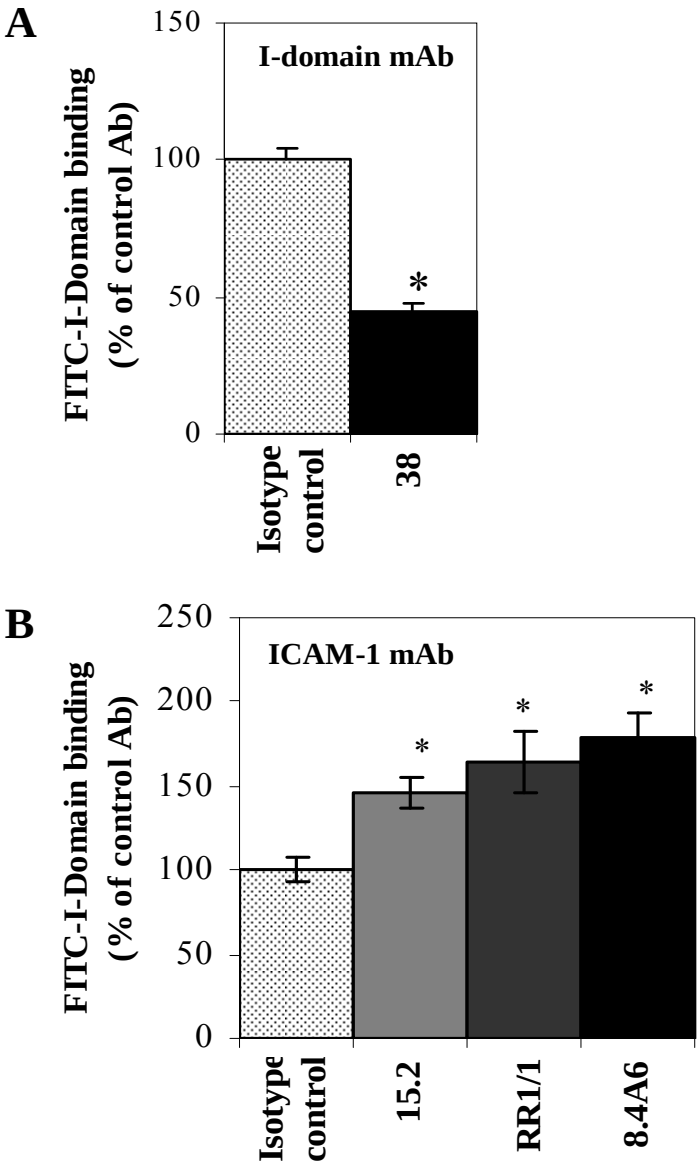


Figure 4. Effect of function blocking mAb [**A.** anti-LFA-1 (CD11a) I-domain mAb and **B.** anti-ICAM-1 (CD54) mAb] on the binding of FITC-I-domain protein to the ICAM-1 receptor expressed on the surface of Raji cells. The results are expressed as the mean ± S.E. (n = 3). There are significant differences in the binding of FITC-I-domain protein; control vs. mAb 38 (*$p<0.01$), RR1/1 (*$p<0.05$), 15.2 (*$p<0.05$), and 8.4A6 (*$p<0.05$).

significantly enhances binding of FITC-I-domain to the cells compared to Mg^{2+} or Mn^{2+} and no divalent cations (Fig. 5). The increase in FITC-I-domain binding could be reversed to normal using Ca^{2+} and EDTA, suggesting the involvement of Ca^{2+} in the MIDAS region of FITC-I-domain during binding to ICAM-1. Finally, the presence of EDTA in Mg^{2+}- or Mn^{2+}-containing media shows binding of FITC-I-domain similar to that of control (absence of cations). These results indicate that Ca^{2+} is a better coordination cation than Mg^{2+} or Mn^{2+} for FITC-I-domain binding to ICAM-1.

3.3.6 FITC-I-domain uptake by Raji cells:

The results of concentration-dependent binding studies suggest that FITC-I-domain binds to the cell surface only at 4 °C; however, a combination of binding and uptake of FITC-I-domain is observed at 37 °C (Fig. 3). To determine the uptake properties, time-dependent binding at 4 °C and 37 °C was studied by flow cytometry (Fig. 6). At 37 °C, 64% of binding was observed within 5 min; 100% binding was arbitrarily assigned to fluorescence intensity at the 360-min time point with a plateau starting at the 30-min point. A three-fold decrease in the fluorescence intensity was found upon 4 °C incubation of the protein, suggesting that the difference in fluorescence intensities at 37 and 4 °C was due to protein uptake.

To visualize the binding and uptake, Raji cells were incubated with FITC-I-domain at either 4 °C or 37 °C with increasing incubation time up to 360 min. This was followed by capturing a confocal Z-section series over a depth of 12-microns. The green fluorescence from the protein could be clearly identified in the cells treated at 37 °C (Figure 7A) as

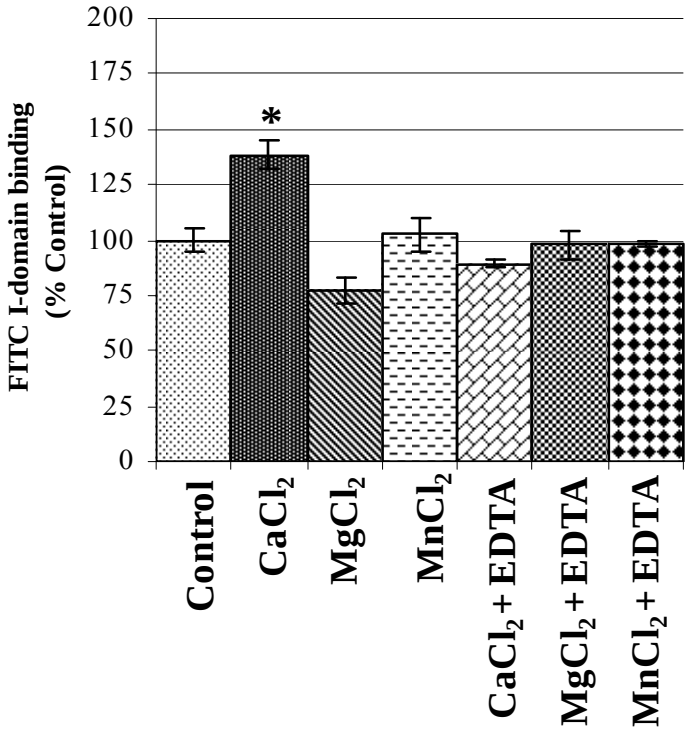


Figure 5. Effect of divalent cations on FITC-I-domain binding to ICAM-1 receptors expressed on the surface of Raji cells. The results are expressed as the mean ± S.E. (n = 3). There are significant differences in the binding of the FITC-I-domain protein; control vs. CaCl2 (*$p<0.05$).

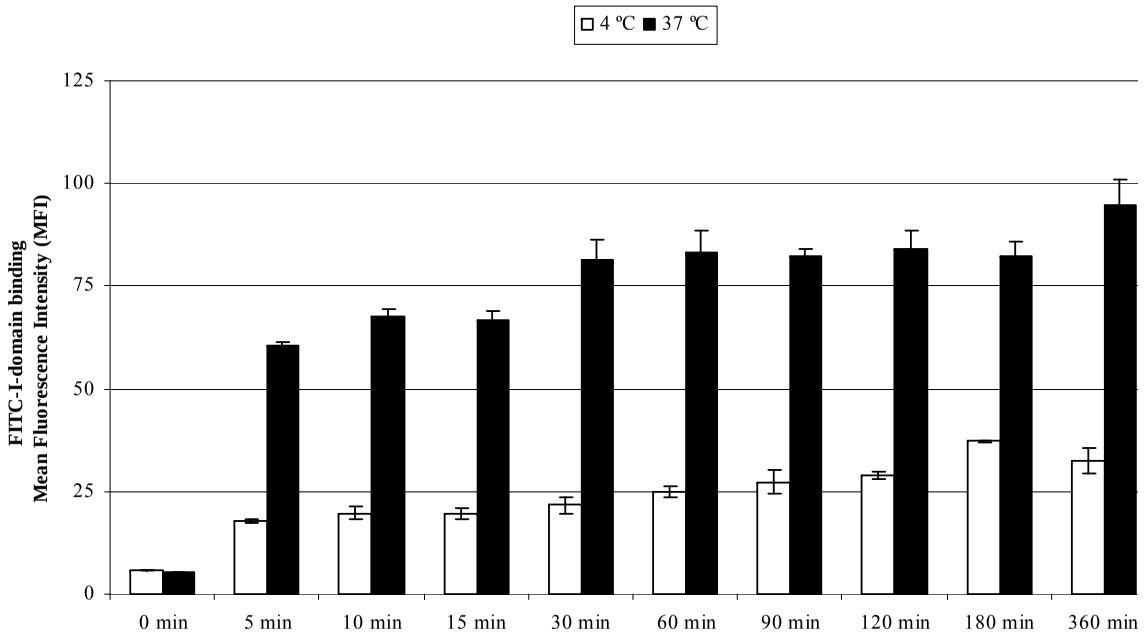


Figure 6. FITC-I-domain protein binding to Raji cells at 37 °C (closed bars) and 4 °C (open bars) as a function of time. The values for the mean fluorescence intensity were obtained using flow cytometry. The results are expressed as the mean ± S.E. (n = 3).

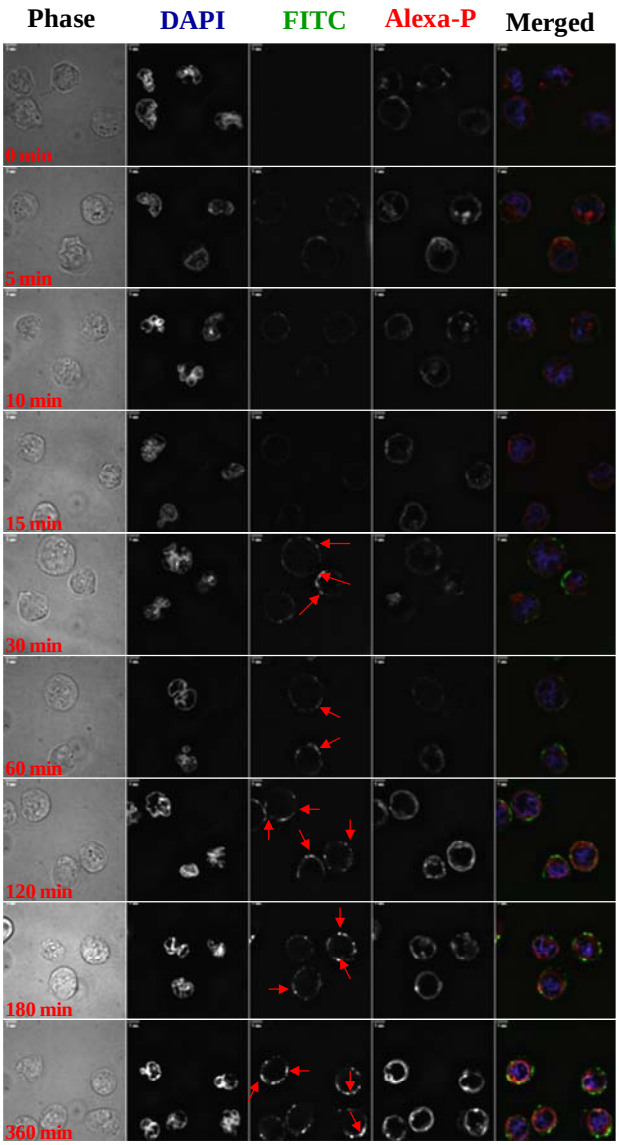


Figure 7A. Confocal microscopy images of FITC-I-domain binding to ICAM-1 on the surface of Raji cells at 37 °C with increasing incubation time (0, 5, 10, 15, 30, 60, 120, 180, and 360 min). Raji cells stained with DAPI (blue) for the nucleus, Alexa Fluor® 647 Phalloidin (red) for actin, and FITC-I-domain (green). Phase contrast image (first row), DAPI (second row), FITC-I-domain (third row), Alexa Fluor® 647 Phalloidin (fourth row), and merged image of all the three colors (fifth row).

early as the 5-min time point. In contrast, only limited green fluorescence was found associated with the cells incubated with the protein at 4 °C (Figure 7B).

To differentiate surface binding and intracellular uptake of FITC-I-domain, the cell nucleus and actin were stained with DAPI and Alexa Fluor® 647 phalloidin, respectively. Using the CellProfiler cell image analysis software, captured images were segmented and processed for green fluorescence from FITC-I-domain associated with regions defined as either the whole cell (i.e., region of DAPI staining plus the region of Phalloidin-Alexa Fluor® 647 staining) or the cytoplasm (i.e., regions of Phalloidin-Alexa Fluor® 647 staining) alone. The average integrated fluorescence intensity (AIF) values associated with the whole cell upon protein incubation at 37 °C were increased upon longer incubation time compared to those incubated at 4 °C; these results were consistent with data from flow cytometry (Fig. 8A). Similarly, the AIF values associated with the cyctoplasm were higher at 37 °C than at 4 °C (Fig. 8B). The AIF intensity values associated with the membrane also increased with incubation time at 37 °C (Fig. 8C). These results indicate that FITC-I-domain conjugate binds and internalizes in B-cells at 37 °C.

3.4 DISCUSSION

This study is the first to show that I-domain can enter the intracellular space of Raji cells *via* a receptor-mediated endocytosis process. Previously, cLABL peptide derived from the sequence of I-domain was found in the intracellular space of T-cells upon binding and ICAM-1-mediated endocytosis.[29] Nanoparticles decorated with cLABL

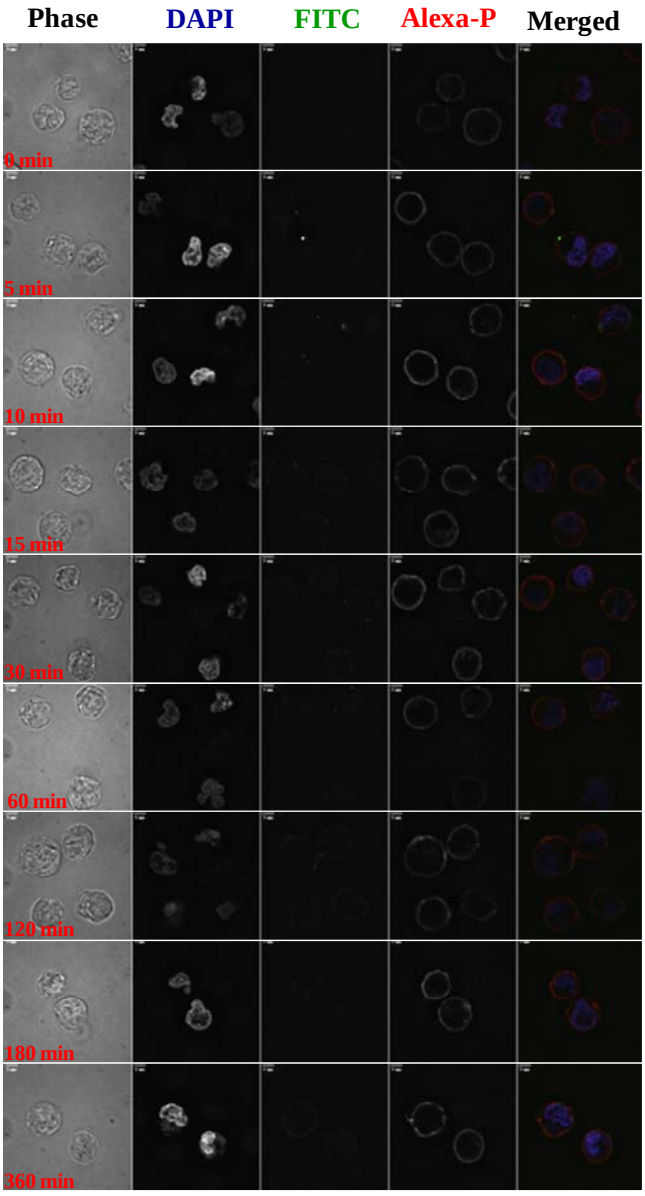


Figure 7B. Confocal microscopy images of FITC-I-domain binding to ICAM-1 on the surface of Raji cells at 4 °C with increasing incubation time (0, 5, 10, 15, 30, 60, 120, 180, and 360 min). Raji cells stained with DAPI (blue) for the nucleus, Alexa Fluor® 647 Phalloidin (red) for actin, and FITC-I-domain (green). Phase contrast image (first row), DAPI (second row), FITC-I-domain (third row), Alexa Fluor® 647 Phalloidin (fourth row), and merged image of all the three colors (fifth row).

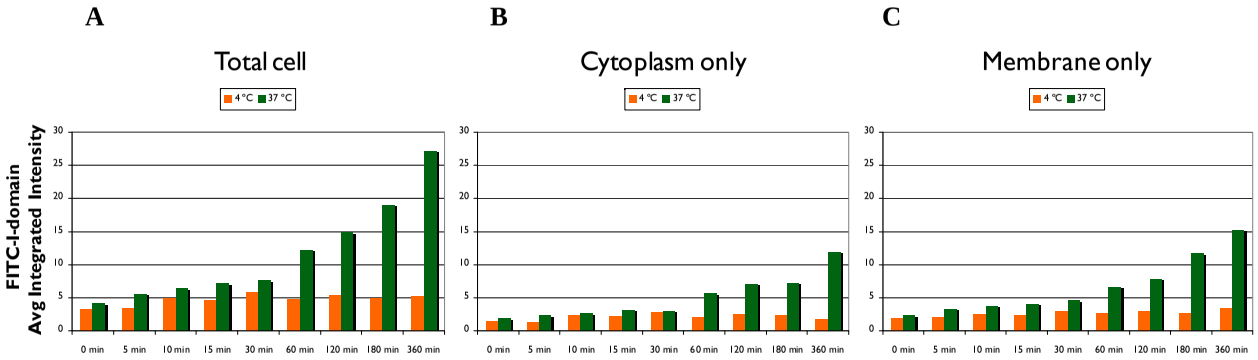


Figure 8. FITC-I-domain protein binding to Raji cells at 37 °C (green bars) and 4 °C (orange bars). The values for the average integrated fluorescence intensities associated with whole cell **(A)**, cytoplasm only **(B)**, and membrane only **(C)** were obtained from the analysis of the confocal images using a cell image analysis software, CellProfiler.

peptide or anti-ICAM-1 antibody were also endocytosed into cellular compartments (*i.e.*, endosomes) upon binding to ICAM-1 on the cell surface.[13, 30-31] Anti-ICAM-1-coated nanoparticles successfully delivered lysosomal enzyme in to cells obtained from patients suffering from lysosomal storage disorder.[32] These nanoparticles are endocytosed *via* a non-classical mechanism upon ICAM-1 clustering, which is called CAM-mediated endocytosis. The CAM-mediated endocytosis is distinct from classical clathrin- or caveolar-mediated internalization as well as from phagocytosis and micropinocytosis processes.[13, 33]

ICAM-1 has important roles in different pathological conditions such as autoimmune diseases,[34-35] cancer,[34, 36] and atherosclerosis.[37] In multiple myeloma, lung, and pancreatic cancer cells, the level of ICAM-1 expression is increased.[34] Upregulation of ICAM-1 is also found on the endothelium during inflammation, which is influenced by cytokines (*i.e.*, TNF-α, IL-1 and IFN-γ), and this upregulation induces adhesion of leukocytes prior to subsequent *trans*-endothelial migration to the injured tissue.[38] Cell surface ICAM-1 molecules serve as endocytosis receptors for human rhinovirus (HRV) and respiratory syncytial virus.[39-40] Thus, the I-domain can be explored as a carrier to target drugs to leukocytes, cancer cells, and inflammatory endothelial cells with up-regulated ICAM-1 receptors. The hope is that the I-domain-drug conjugate can direct the drug molecule to the target pathogenic cells while at the same time avoiding normal cells to minimize the toxic side-effects of the drug. The small drugs can be conjugated to several of the 20 Lys residues of the I-domain. Here, several of these Lys residues of the I-domain were conjugated with FITC groups and still maintained the I-domain conformation (Fig. 1C),

ICAM-1-binding (Fig. 3), and uptake properties (Figs. 6–8). We have determined using tryptic mapping, mass spectrometry, and molecular modeling that the FITC-conjugated Lys residues are away from the binding site of I-domain to ICAM-1.

It is clear that FITC-I-domain binds to ICAM-1 on Raji cells and that this binding can be inhibited by anti-LFA-1 mAb to I-domain clone 38, suggesting that the antibody blocks the I-domain binding site to ICAM-1 (Fig. 4). However, all anti-ICAM-1 mAb enhance the binding of FITC-I-domain (Fig 4), indicating that the I-domain has a different binding site on ICAM-1 than does these mAb. A similar effect of RR1/1 mAb was observed in the binding of a GST-tagged I-domain (I-GST) to a surface-coated ICAM-1Fc using a solid-phase ELISA assay. This result also suggests that the I-domain protein interacts with Glu-34 of ICAM-1 but not Gln-73.[26] There are several possible explanations for the enhancement of I-domain binding. First, anti-ICAM-1 mAb binding may induce conformational change in a single ICAM-1 to an active conformation for binding to the I-domain. Second, anti-ICAM-I mAb could promote dimerization or oligomerization (clustering) of the ICAM-1 on the cell surface that binds to multiple regions of a single I-domain molecule. Finally, the mAb can induce ICAM-1 conformational change to promote dimers or cluster for binding to multiple sites in an I-domain molecule to increase the binding affinity. The antibody studies indicate that FITC-I-domain binds to ICAM-1 on Raji cells, and the presence of FITC labels does not alter its binding to ICAM-1.

The binding of FITC-I-domain to ICAM-1 is enhanced by Ca^{2+} ion but not Mg^{2+} or Mn^{2+} ions (Fig. 5); this increase in binding can be reversed upon addition of EDTA. It is

interesting to that only Ca^{2+} ion enhances the binding of FITC-I-domain to ICAM-1; this could be due to the fact that Ca^{2+} has the appropriate size and electrostatic interaction compared to Mg^{2+} and Mn^{2+} to coordinate with the MIDAS region of the I-domain and the Glu-34 residue of ICAM-1. Mn^{2+} strongly augmented the binding of LFA-1 expressed on the surface of T-cells to its ligand, ICAM-1,[41] suggesting that Mn^{2+} is able to induce conformational changes in LFA-1 favoring the high affinity state. Binding of Mg^{2+} with in the I-domain also results in a conformational change such that the C-terminal α7 helix swings away from the central β-sheet, resulting in a high affinity form of I-domain.[42]

Receptor-mediated internalization, along with other technological advances in drug conjugation, may be a useful application for I-domain in targeting drugs to inflammatory diseases, autoimmune diseases, and cancer. Recently, we have successfully conjugated an antigenic peptide with an I-domain protein via a linker. The antigenic peptide-I-domain conjugate effectively suppressed experimental autoimmune encephalomyelitis (EAE) in female SJL/J mice as a model for human multiple sclerosis (Chapter 4).

We believe that FITC-I-domain binding to ICAM-1 on the cell surface induces ICAM-1 clustering, further initiating the internalization of the conjugate via an ATP-dependent process. We speculate that this is CAM-mediated endocytosis. The binding sites on ICAM-1 for HRV and LFA-1 are distinct with partial overlaps in the binding residues. Studies done using ICAM-1 mutants revealed that the binding site for HRV on ICAM-1 is Gln 58, while for LFA-1 is Glu 34.[27] Antibodies can selectively bind to the target receptor, but it is not guaranteed that such interactions necessarily result in

endocytosis. *In vitro* and *in vivo* animal studies done using anti-ICAM-1 have shown that the ICAM antibody internalizes poorly.[43] In contrast, targeting using LFA-1 I-domain-conjugates is mediated by the comparatively natural interaction between the I-domain and its ligand ICAM-1 receptor. Therefore, I-domain conjugates may offer an alternate solution for selective and effective internalization.

ICAM-1 targeting offers a variety of applications. ICAM-1-targeted isotopes were used for detection of lung inflammation at a very early stage.[44] Liposomes conjugated with anti-ICAM-1 were able to bind to epithelial and endothelial cells in a dose- and time-dependent manner.[45] Murciano *et al.* demonstrated the delivery of an anti-ICAM-1 conjugated tissue-type plasminogen activator (tPA) to endothelial cells *in vitro* and in an *in vivo* animal model[43] Thus, ICAM-1 targeting has been utilized for imaging, diagnosis, and delivery of therapeutic agents.

3.5 CONCLUSIONS

In this chapter, we have shown that the FITC-I-domain can bind to ICAM-1 on the surface of B-cells. The conjugate binding is influenced by the presence of calcium. Further, the conjugate is internalized by ICAM-1 on B-cells, probably via receptor-mediated endocytosis, suggesting that the I-domain can be used to target drugs to the cytoplasmic compartment of cells expressing ICAM-1. In the future, the utility of I-domain proteins in delivering anti-inflammatory, anti-cancer, and antigenic peptides will be investigated.

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CHAPTER 4

In vivo Suppression of EAE by PLP-I-domain Conjugates

4.1 INTRODUCTION

Autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and type 1 diabetes (T1D) are caused by host immune systems that recognize self tissues or organs. In the case of MS, the immune systems attack the myelin sheath of the neurons causing disruption of the signal translation in the central nervous system (CNS). One of the potential ways that T cells recognize the myelin sheath is by activation of a subset of autoreactive T cells to recognize the self myelin sheath. One possible mechanism of activation of a subpopulation T cell is via formation of the "immunological synapse" at the interface between T cells and antigen-presenting cells (APC). The immunological synapse is a "bull's eye"-like structure that is composed of a cluster of interactions between T cell receptors (TCR) and major histocompatibility complex-peptide (MHC-p) at the center (Signal-1) and a cluster of interactions between costimulatory molecules (i.e. Signal-2, B7/CD28, ICAM-1/LFA-1) at the periphery of the bull's eye. The differentiation of naïve T cells to a specific subset (i.e. T_H1, T_H2) is strongly dependent on the type of co-stimulatory signal being delivered. Blocking Signal-2 during this process leads to immune unresponsiveness of T cells called anergy.[1] Inhibition of ICAM-1/LFA-1 interaction suppresses T_H1-type immune response and promotes a non-inflammatory T_H2-type response.[2] Potential drugs such as monoclonal antibodies (mAb) or small molecules have been developed based on blocking Signal-2. Drugs for autoimmune diseases that inhibit Signal-2 may also suppress the general immune response of the host; unfortunately, as a potential side effect, these drugs may compromise the ability of the host to respond to pathogenic infections.

To overcome the general suppression of immune systems, our approach is to modulate the activation of a subpopulation of T cells that recognizes a specific antigen using a bifunctional peptide inhibitor (BPI). The BPI molecules are composed of an antigenic peptide for the specific disease conjugated to a cell adhesion peptide via a spacer.[3-7] GAD-BPI, PLP-BPI, and CII-BPI molecules have been shown to induce immunotolerance in non-obese diabetes (NOD),[5] experimental allergic encephalomyelitis (EAE),[3-4, 6-7] and collagen-induced arthritis (CIA), respectively. The antigenic peptide is derived from the sequence of antigenic epitope responsible for the autoimmune disease while the adhesion peptide is derived from either the sequence of LFA-1 (LABL) or ICAM-1 (cIBR7). The potential mechanism of action of BPI molecules is via simultaneous binding to MHC-II and ICAM-1 receptors on the surface of APC, respectively. Simultaneous binding to these two target receptor prevents the translocation Signal-1 and Signal-2 molecular complexes and inhibits the formation of the immunological synapse. As a result, the BPI molecules suppress the generation of inflammatory T cells and possibly stimulate the formation of suppressor or regulatory T cells.

In this work, we have designed PLP-I-domain-1 and -2 molecules as potential therapeutic agents for the treatment of multiple sclerosis. PLP-I-domain was made by conjugating $PLP_{139-151}$ peptide via a maleimide spacer to several lysine residues on the I-domain protein derived from the α-subunit of LFA-1. The I-domain is the binding region of LFA-1 to ICAM-1, and it has been shown to interact with the D1 domain of ICAM-1.[8] Compared to the cell adhesion peptide LABL, I-domain offers a unique divalent cation

coordination site called metal ion-dependent adhesion site or MIDAS that interacts with the ICAM-1 D1 domain. Furthermore, there are potential multiple conjugation sites on the I-domain. In this study, PLP-Cys-OH and PLP-Cys-NH_2 were conjugated to γ-maleimidobutyryloxy-I-domain (GMB-I-domain) at pH 8.5 and 6.6 to give PLP-I-domain-1 and PLP-I-domain-2, respectively. The efficacies of PLP-I-domain-1 and -2 were compared to those of PBS, I-domain, GMB-I-domain as negative controls, and Ac-PLP-LABL-NH_2 and Ac-PLP-cIBR7-NH_2 as positive controls. The results show that the difference in *in vivo* activity of PLP-I-domain-1 and PLP-I-domain-2 was due to the conjugation sites of the two PLP-I-domain molecules.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The amino acids used for peptide synthesis were purchased from Peptide International (Louisville, KY). GMBS (*N*-[γ-maleimidobutyryloxy]succinimide ester) was from Pierce (Rockford, IL). Sequence-grade modified trypsin was from Promega (Madison, WI). All other chemicals or solvents were of analytical grade or better.

4.2.2 Mice

The *in vivo* studies were carried out using female inbred SJL/J (H-2^S) mice purchased from Charles River Laboratories, Inc. (Wilmington, MA). The animals were housed under specific pathogen-free conditions at an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility at the University of Kansas. All protocols involving live mice were pre-approved by the university's Institutional Animal Care and Use Committee (IACUC) and are in compliance with the committee's recommendations.

4.2.3 Peptide synthesis

The sequences of peptides used in the present study are listed in Table 1. The peptides were synthesized by standard Fmoc solid-phase peptide chemistry on appropriate PEG-PS™ resin (Applied Biosystems, Foster City, CA) using the automated peptide synthesis system (Pioneer™ perspective Biosystems, Framingham, MA). Peptide synthesis and purification was conducted according to our previously published method.[4] All peptides were purified using semi-preparative C_{18} reversed-phase HPLC, and the fractions from the preparative HPLC were analyzed by analytical HPLC. The pure fractions of the peptide were pooled and lyophilized; the molecular weight of the peptides was confirmed by electrospray ionization mass spectrometry (M+1) (MW PLP-Cys-OH = 1624.86; MW PLP-Cys-NH_2 = 1623.81; MW Ac-PLP-BPI-NH_2 = 3416.95; MW Ac-PLP-cIBR-NH_2 = 3093.79).

Table 1. List of peptides and proteins used in the present study	
Peptide/Protein	**Sequence**
Ac-PLP-LABL-NH_2	*Ac*-HSLGKWLGHPDKF-$(AcpGAcpGAcp)_2$-ITDGEATDSG-*NH_2*
Ac-PLP-cIBR7-NH_2	*Ac*-HSLGKWLGHPDKF-$(AcpGAcpGAcp)_2$-cyclo(1,8)CPRGGSVC-*NH_2*
PLP-I-domain-1	$(HSLGKWLGHPDKFC)_n$-linker-I-domain
PLP-I-domain -2	$(HSLGKWLGHPDKFC\text{-}NH_2)_n$-linker-I-domain
GMB-I-domain	[*N*-(γ-maleimido)-1-oxybutyl]$_n$-I-domain
I-domain	MGNVDLVFLFDGSMSLQPDEFQKILDFMKDVMKKLSN TSYQFAAVQFSTSYKTEFDFSDYVKRKDPDALLKHVK HMLLLTNTFGAINYVATEVFREELGARPDATKVLIIITD GEATDSGNIDAAKDIIRYIIGIGKHFQTKESQETLHKFAS KPASEFVKILDTFEKLKDLFTELQKKIY
Ac = Acetyl and Acp = Aminocaproic acid	

4.2.4 Preparation of I-domain

The LFA-1 I-domain protein was over-expressed, refolded, and purified as previously described.[9] The protein purity, identity, and secondary structure were confirmed by SDS-PAGE, mass spectrometry, and far-UV circular dichroism (CD), respectively.

4.2.5 Synthesis of PLP-I-domain-1 and -2

Two steps are required to prepare the PLP-I-domain conjugates. The first is to modify the amino groups of the N-terminal and side-chain of lysine residues of I-domain by reacting them with *N*-[γ-maleimidobutyryloxy]succinimide ester (GMBS). This step introduces maleimido groups on the I-domain to generate the GMB-I-domain. The second step is to conjugate the thiol group on the Cys residue of PLP-Cys-OH and PLP-Cys-NH_2 peptides to the maleimide groups on the I-domain to give PLP-I-domain-1 and PLP-I-domain-2, respectively.

4.2.5.1 Step 1: To a total of 20 mg of I-domain solution, a tenfold molar excess of freshly prepared GMBS (2.71 mg) solution in DMSO (0.5 mL) was added dropwise followed by stirring of the mixture for 1 h at 24 °C. Then, the reaction mixture was subjected to purification through a Superdex 75 column to isolate the GMB-I-domain. The desired GMB-I-domain and the excess GMBS were eluted with PBS containing 10 mM $MgSO_4$. The fractions containing the GMB-I-domain were collected and concentrated by ultrafiltration. Modification on the I-domain using this method gave 3–10 maleimido groups per I-domain as determined by electrospray ionization mass spectrometry (ESI-MS).

4.2.5.2 Step 2: The conjugation reactions of PLP-Cys-OH and PLP-Cys-NH_2 peptides to GMB-I-domain were carried out at pH 8.5 and 6.6, respectively, to produce PLP-I-domain-1 and PLP-I-domain-2. To a solution containing 10 mg of GMB-I-domain, a 15 molar excess of PLP-Cys-OH or PLP-Cys-NH_2 dissolved in PBS was added dropwise. During the addition of the peptide, the pH was constantly monitored and adjusted to either 8.5 or 6.6, depending on the reaction. During the reaction, the final concentration of the protein in both batches was 2.0 mg/mL. The reaction was carried out for 1 h at 24 °C with constant stirring. After the reaction was complete, the pH of the mixture was readjusted to 7.4. The resulting PLP-I-domain-1 or PLP-I-domain-2 was purified using a Superdex 75 column. The fractions belonging to the PLP-I-domain-1 or -2 were collected and concentrated by ultrafiltration. Both PLP-I-domain-1 and -2 contained 0 to 5 peptide molecules per I-domain molecule as determined by ESI-MS. The purity of the PLP-I-domain-1 and -2 was confirmed by SDS-PAGE gel and size-exclusion chromatography. The CD spectra of the PLP-I-domain-1 and -2 were compared with that of the parent I-domain protein.

4.2.6 Gel electrophoresis

100 μg of pure protein solution of PLP-I-domain-1, PLP-I-domain-2, or I-domain obtained after SEC separation was mixed with a 4X Tris–glycine SDS sample buffer containing no reducing agent and loaded into 1.5-mm-thick 10-well NuPAGE® Novex 4–12% Bis-Tris gradient gels. After running gel electrophoresis at 150 V for 70 min, the gels were stained with 0.25% Coomassie blue R250 solution (10% acetic acid/50%

ethanol/40% water) for 30 min followed by destaining (10% acetic acid/25% ethanol/65% water) until the bands were visible and the background was clear.

4.2.7 In-Gel trypsin digestion

A standard in-gel protein digestion protocol was followed as described elsewhere.[10] Briefly, protein bands were excised from the gel and were digested with trypsin at an enzyme-to-substrate ratio of 1:25 (w/w) at 37 °C overnight. To stop the digestion, 2 μL of glacial acetic acid was added to each sample.

4.2.8 LC-MS/MS analysis of tryptic-digest products

The products of tryptic digest from I-domain, PLP-I-domain-1, and PLP-I-domain-2 were introduced into a capillary reversed-phase LC-MS/MS using a tandem LTQ-FT mass spectrometer (ThermoFinnigan, Bremen, Germany) under conditions described previously.[11] The experimental raw data were processed using Bioworks software (Thermo, version 2.0) to create an MS/MS peak list in a DTA format. Protein sequence mapping was performed using Sequest, Mascot (Matrix Science, version 2.2) and X!Tandem (www.thegpm.org) algorithms with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 1.2 Da. Amino groups of lysine residues and protein N-terminus were considered to be modified with maleimide linker moiety + dipeptide (Phe-Cys). The chemical composition of the modification for PLP-I-domain-1 is $C_{20}H_{23}N_3O_6S$, delta monoisotopic mass 433.1308 and its maleimide hydrolysis product is $C_{20}H_{25}N_3O_7S$, delta monoisotopic mass 451.1413. The chemical composition of the modification for

PLP-I-domain-2 is $C_{20}H_{24}N_4O_5S$, delta monoisotopic mass 432.1467 and its maleimide hydrolysis product is $C_{20}H_{26}N_4O_6S$, delta monoisotopic mass 450.1572. The assumption is that the trypsin digestion cleaves at the carboxyl side of the lysine residue (^{12}K) of the peptide PLP-Cys-OH or PLP-Cys-NH_2. Scaffold software (Proteome Software Inc., version 2.06) was used to combine and validate MS/MS-based peptide identifications. Peptide identifications with greater than 50% probability as specified by the Peptide Prophet algorithm[12] were accepted for reporting protein coverage.

4.2.9 Induction and suppression of EAE

Female inbred SJL/J mice, 5-7 weeks old, were randomly divided into seven groups. All mice were immunized with $PLP_{139–151}$ in CFA to induce EAE, as reported previously.[3-4] The PLP/CFA emulsion (50 μL per site) was administered to four separate regions above the shoulder and on the flanks. In addition, 200 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was injected intraperitoneally on day 0 and 2. Then, the mice received intravenous injections of either vehicle (PBS), PLP-I-domain-1 (26 nmol/injection), PLP-I-domain-2 (26 nmol/injection), GMB-I-domain (26 nmol/injection), I-domain (26 nmol/injection), or Ac-PLP-cIBR7-NH_2 (52 nmol/injection) on days 4 and 7. The Ac-PLP-LABL-NH_2-treated mice received 100 nmol/injection on days 4, 7, and 10. The animals were weighed and observed daily. Disease progression was evaluated observed using a blinded method as reported previously.[3-4] The clinical scores were rated using the following scale: 0–no clinical signs of disease; 1–tail weakness or limp tail; 2–paraparesis (weakness or incomplete

paralysis of one or two hind limbs); 3–paraplegia (complete paralysis of two hind limbs); 4–paraplegia with forelimb weakness or paralysis; and 5–moribund or dead. Mice were euthanized once they were found to be moribund.

4.2.10 Statistical analysis

Statistical differences among the groups in clinical disease scores were determined by calculating the average score for each mouse from the day of disease onset to day 20 by One-way Analysis of Variance followed by Fisher's least significant difference using StatView (SAS Institute, Cary, NC). Statistical differences among the groups in body weight were also analyzed in the same way. The presence of significant difference is denoted with *p*-values of < 0.05 or < 0.001.

4.3 RESULTS

4.3.1 Synthesis and characterization of PLP-I-domain:

PLP-I-domain-1 and -2 were prepared by conjugating the PLP-Cys-OH and PLP-Cys-NH_2 peptides to the N-terminus and side chain amino groups of the lysine residues in the I-domain (Fig. 1). Thus, the amino groups in the I-domain were reacted with the active N-hydroxysuccinimide (NHS) ester of GMBS to produce GMB-I-domain protein via a stable amide bond (Step 1, Fig.1). The GMB-I-domain from the reaction mixture was purified from the excess GMBS using SEC (Fig. 2A). Comparison of CD spectra of

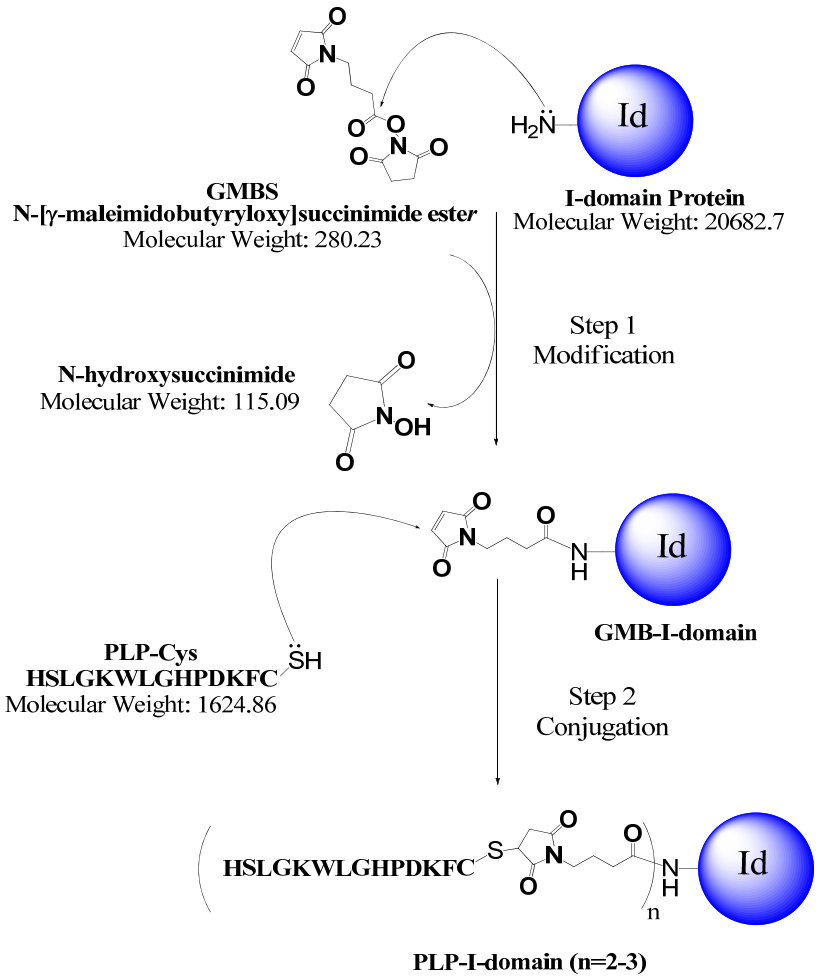

Figure 1. Schematic representation of two-step modification and conjugation reactions to prepare PLP-I-domain-1 and -2.

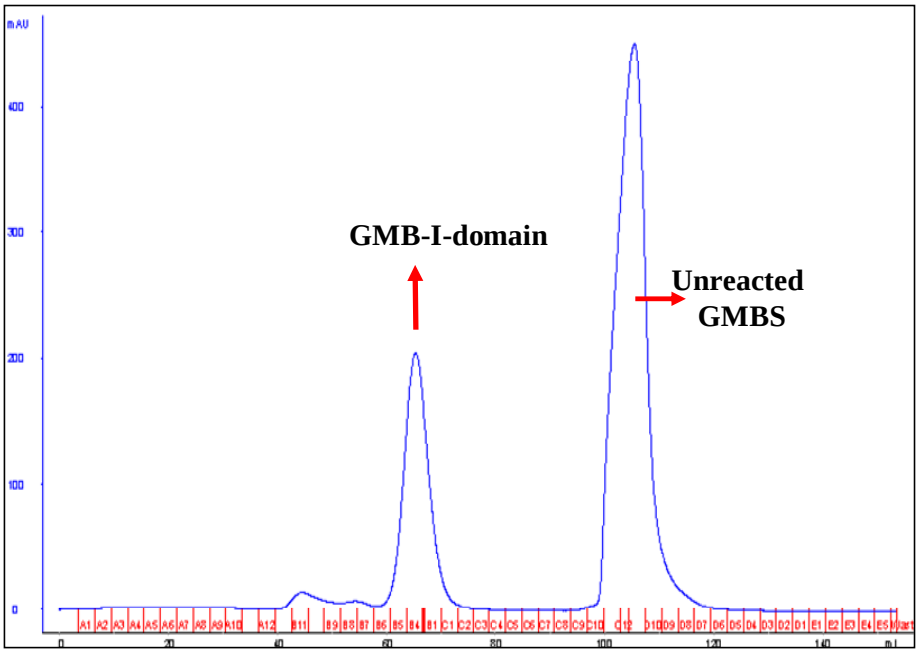

Figure 2A. SEC chromatogram showing the separation between GMB-I-domain and the remaining free GMBS.

GMB-I-domain and I-domain shows that they have similar spectra (Fig. 2B), indicating that adding of GMB groups does not alter the secondary structure of the GMB-I-domain.

The composition of pure GMB-I-domain was analyzed by liquid chromatography coupled online with ESI-MS. The deconvoluted MS spectrum shows three to nine GMB groups attached to the I-domain with following masses: 21,178 Da, 21,343 Da, 21,508 Da, 21,674 Da, 21,839 Da, 22,004 Da, and 22,169 Da (top panel, Fig. 2C). The sequential differences in mass are 165 Da, which is consistent for a sequential addition of GMB group. The first peak at 21,178 Da corresponds to the I-domain molecular weight conjugated to three GMB groups; therefore, the remaining peaks correspond to the I-domain with four to nine covalently linked GMB groups, respectively. The parent I-domain with a MW of 20,682 was not found in the MS spectrum (bottom panel, Fig. 2C).

Along with the desired GMB-I-domain peak, there are corresponding peaks with a mass increase of 18 Da found in the MS spectra (top panel, Fig. 2C). These peaks correspond to the hydrolysis of maleimide groups or maleic acid derivatives, which are attached to the I-domain.[13] The MS data correlate with the SDS-PAGE image of the isolated product of GMB-I-domain from SEC, which shows two bands on lane 4 in Figure 2D. These two bands are from the desired maleimide and maleic acid derivatives of I-domain with different electrophoretic mobility. Before purification, the reaction mixture (lane 3, Fig. 2D) shows three bands while the control I-domain (lane 2, Fig. 2D) produces only one band. The formation of maleimide hydrolysis products is increased upon storage; thus, it is important that the GMB-I-domain be used within 48 h after SEC purification.

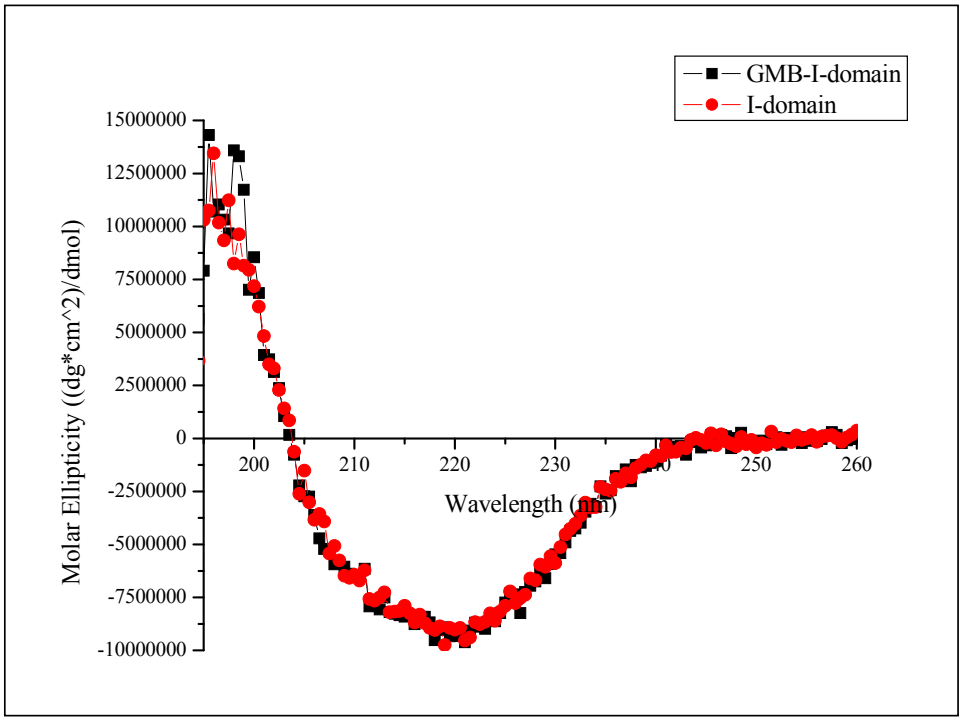

Figure 2B. CD spectra of the parent I-domain (red) and GMB-I-domain (black).

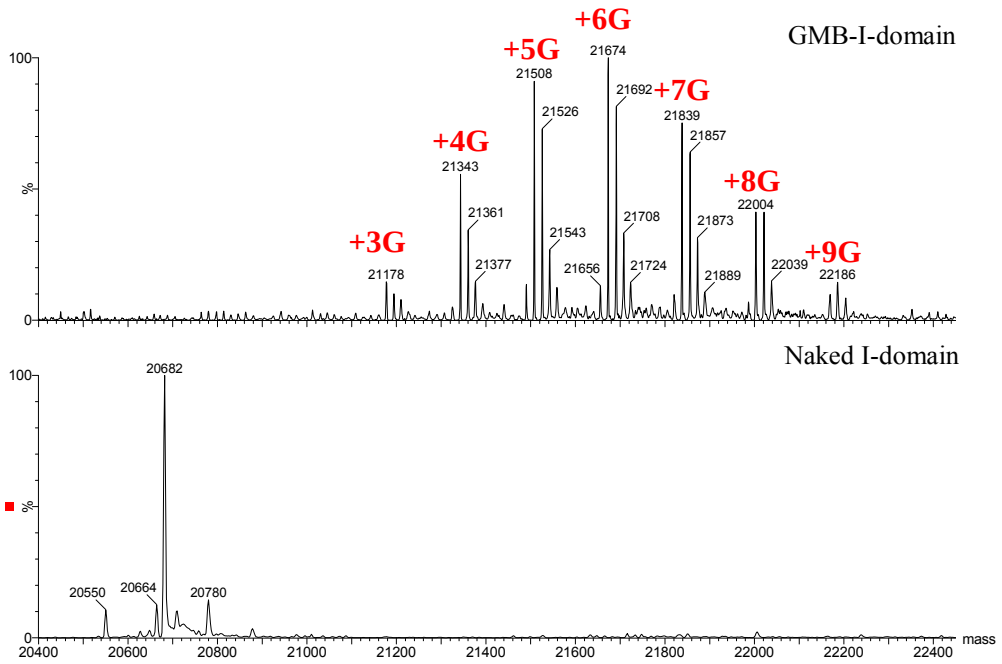

Figure 2C. Deconvoluted mass spectra of LC ESI-MS analysis of the GMB-I-domain protein (top) and the parent I-domain protein (bottom).

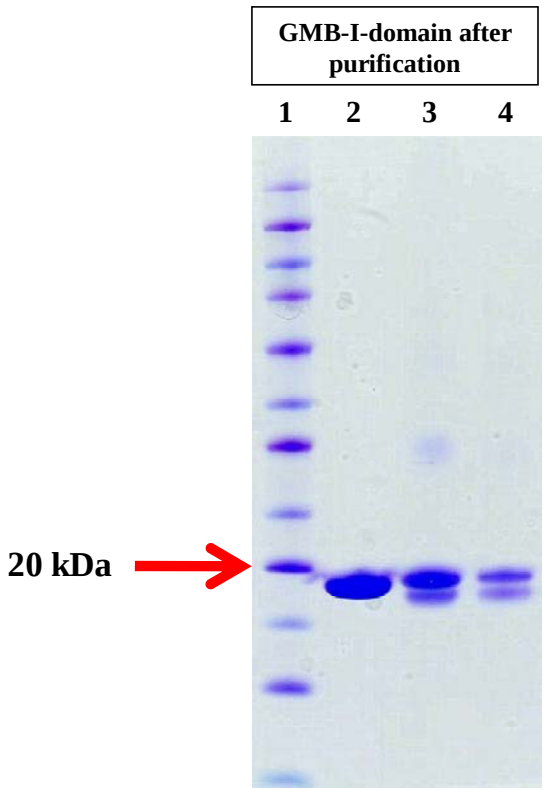

Figure 2D. SDS-PAGE analysis of pure GMB-I-domain protein after staining with Coomassie blue: molecular weight marker (lane1), the I-domain protein (lane 2), the reaction mixture of I-domain protein and GMBS (lane 3), and GMB-I-domain protein (lane 4).

4.3.2 Conjugation of PLP-Cys-OH and PLP-Cys-NH_2 peptide to GMB-I-domain:

PLP-Cys-OH and PLP-Cys-NH_2 are peptides that contain $PLP_{139-151}$ sequence with an additional cysteine amino acid at the C-terminus. PLP-Cys-OH has open carboxylic acid (Cys-OH) and PLP-Cys-NH_2 has amidated carboxylic acid (Cys-NH_2) at the C-terminus. PLP-Cys-OH and PLP-Cys-NH_2 peptides were reacted with the GMB-I-domain at pH 8.5 and 6.6 to make PLP-I-domain-1 and PLP-I-domain-2, respectively. In this case, the peptide conjugation is via nucleophilic attack of the maleimide groups on the GMB-I-domain by the thiol group of the Cys residue on the peptide (Step 2, Fig. 1). Both crude products were purified by SEC; the desired PLP-I-domain-1 or -2 could be easily separated from PLP-Cys-OH or PLP-Cys-NH_2 (Fig. 3A). The pure fractions of each PLP-I-domain were pooled and concentrated. The crude and purified products were analyzed by SDS-PAGE gel against the parent I-domain (Fig. 3B). As expected, the parent I-domain shows one single band with low molecular weight than the conjugates (lane 2, Fig. 3B). The gel of the crude product illustrates the presence of PLP-I-domain-1 or -2 along with lower MW bands corresponding to PLP-Cys-OH or PLP-Cys-NH_2, respectively (lanes 3 and 5, Fig 3B). The gel of purified PLP-I-domain-1 or -2 shows multiple bands on the gel with higher molecular weight than the parent I-domain and without the starting PLP-Cys-OH or PLP-Cys-NH_2 (lanes 4 and 6, Fig. 3). The multiple bands on PLP-I-domain-1 and -2 were due to various levels of peptide conjugates. Using the gel visually, it is difficult to differentiate between PLP-I-domain-1 and -2.

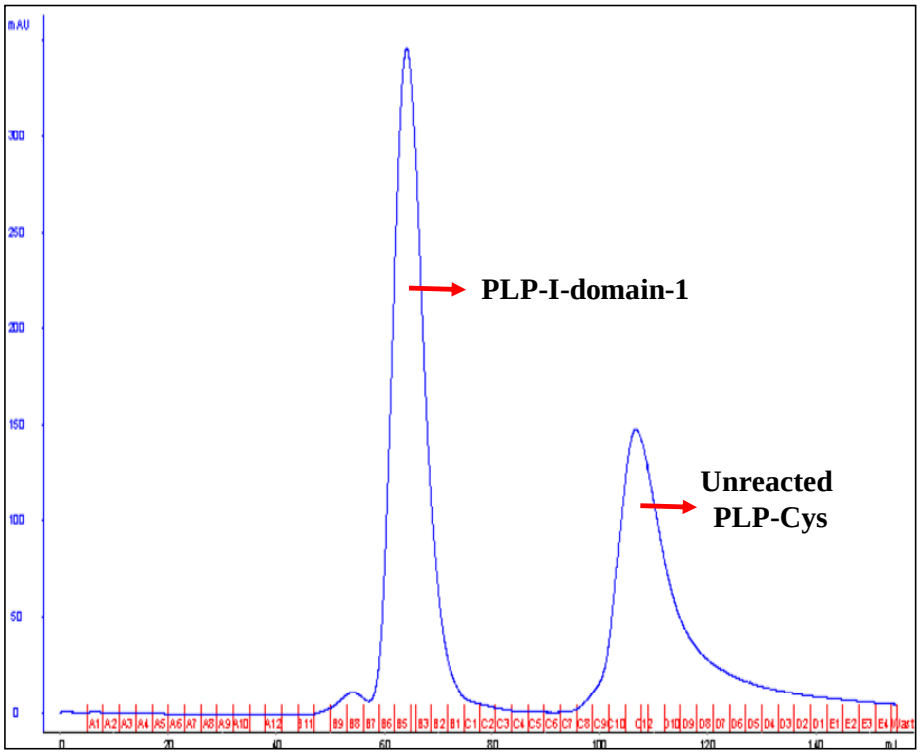

Figure 3A. The SEC chromatogram of PLP-I-domain-1, which is separated from the PLP-Cys peptide.

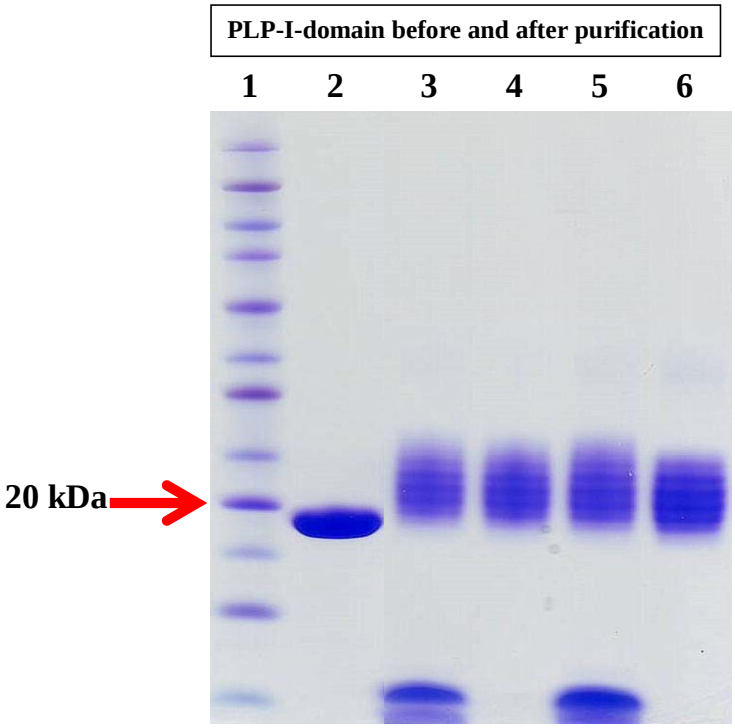

Figure 3B. SDS-PAGE analysis of different proteins after staining with Coomassie blue: molecular weight marker (lane1), the parent I-domain protein (lane 2), the reaction mixture at pH 8.5 to make PLP-I-domain-1 (lane 3), the purified PLP-I-domain-1 (lane 4), the reaction mixture at pH 6.6 to make PLP-I-domain-2 (lane 5), and the purified PLP-I-domain-2 (lane 6).

The deconvoluted LC-MS data indicated that 0–5 PLP peptides were attached to the PLP-I-domain-1 or -2 with an average of 2.5 PLP peptides per I-domain (Fig. 3C). Each subpopulation of peaks in the spectra of PLP-I-domain-1 or -2 has various MW peaks due to the presence of different numbers of GMB groups, but the same number of PLP-Cys peptide attached to the I-domain. The complexity in each of the subpopulations arises from the hydrolysis products of maleimide Figure 4.[13] The CD spectrum of each conjugate was similar to that of the parent I-domain protein (Fig. 3D), indicating that conjugation of PLP-Cys peptide to the I-domain preserves the native secondary structure of the protein.

4.3.3 Prophylactic suppression of EAE by PLP-I-domain-1 and -2:

The *in vivo* efficacies of PLP-I-domain-1 and -2 to suppress EAE were determined in SJL/J mice (Fig. 5). After PLP/CFA immunization of the mice on day 0, they were treated via i.v. injections with PLP-I-domain-1 or -2, negative controls (PBS, I-domain, GMB-I-domain), or positive controls (Ac-PLP-cIBR7-NH_2 and Ac-PLP-LABL-NH_2). The mice treated with PBS, I-domain, and GMB-I-domain showed disease signs of EAE from days 8 to 17 after immunization with clinical scores as high as 4 (Fig. 5A). Similar trends were observed in loss of body weight of animals treated with these negative controls (Fig. 5B). At day14, 95% or more of the mice in these three groups had EAE (Fig. 5C). Both positive controls were significantly more effective in suppressing EAE than was to PBS ($p < 0.001$ for clinical scores and body weights). Ac-PLP-cIBR7-NH_2

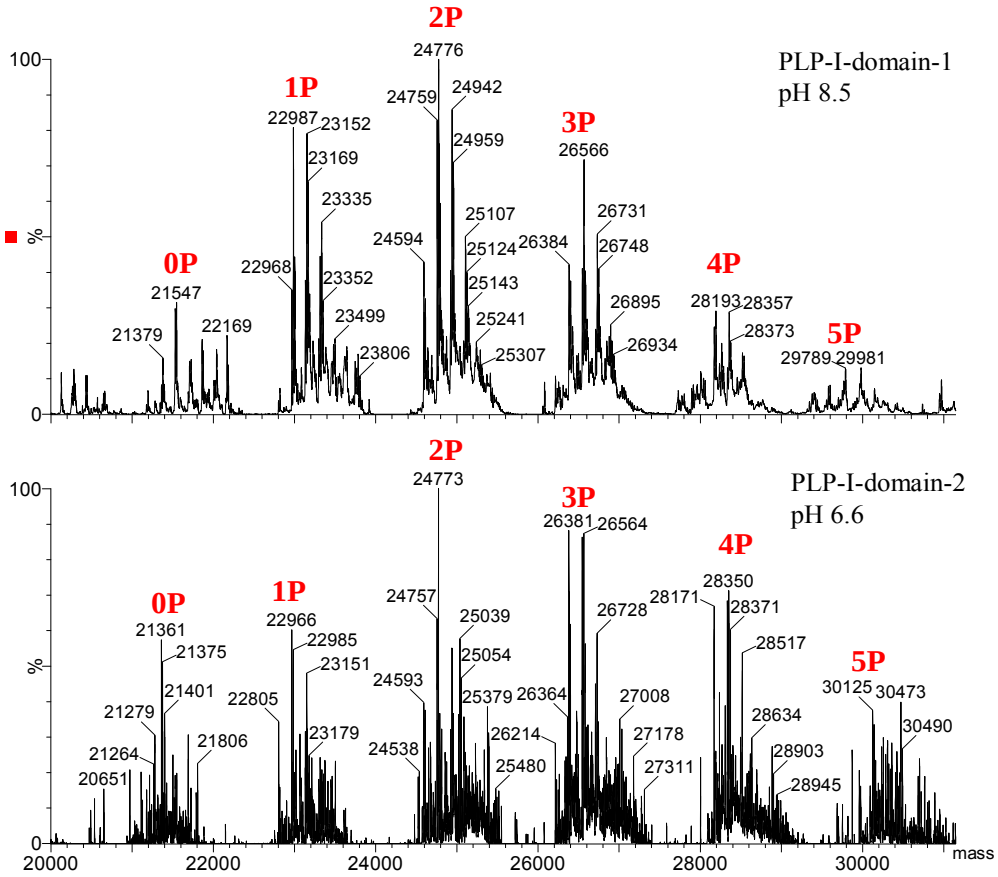

Figure 3C. Deconvoluted mass spectra of LC ESI-MS analysis of the PLP-I-domain-1 preparation (top) and PLP-I-domain-2 (bottom).

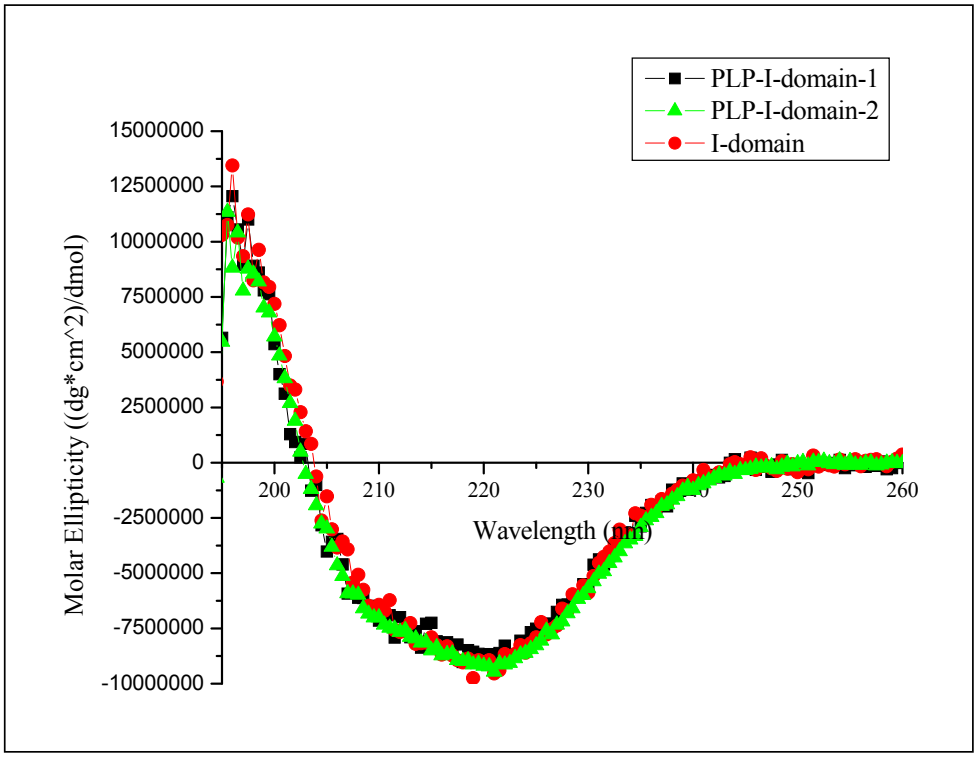

Figure 3D. CD spectra of the parent I-domain (red), PLP-I-domain-1 (black) and PLP-I-domain-2 (green).

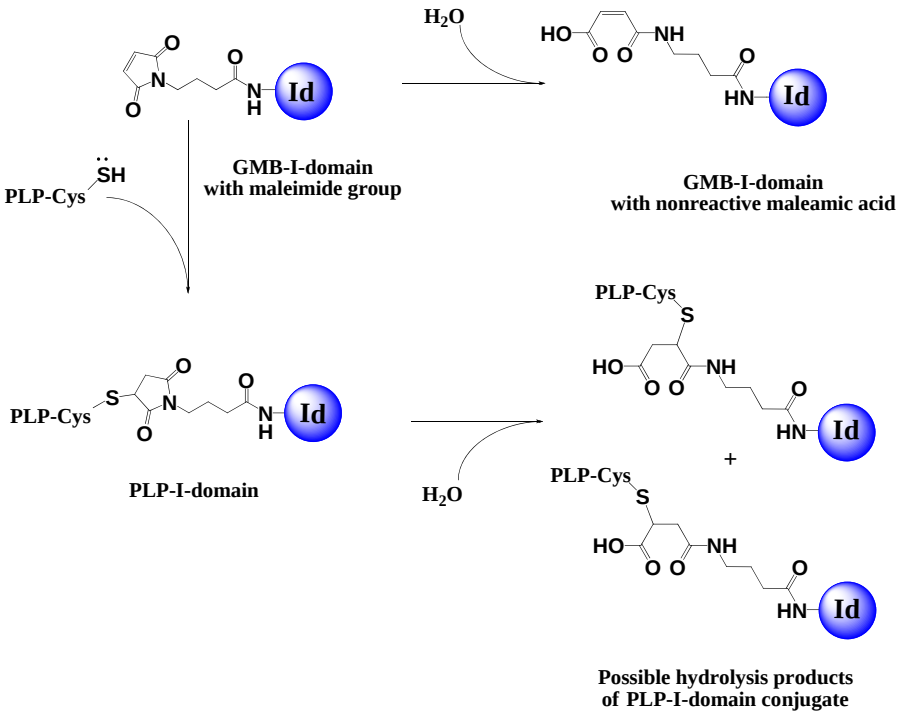

Figure 4. Schematic representation of possible hydrolysis products of GMB-I-domain and PLP-I-domain conjugates.

had a better efficacy than Ac-PLP-LABL-NH_2 with the onset of the disease delayed by 7 and 4 days, respectively (Fig. 5A).

Mice treated with PLP-I-domain-1 have significantly lower EAE clinical scores (Fig. 5A) and minimal or no loss of body weight (Fig. 5B) compared to mice treated with PBS ($p < 0.001$), I-domain, and GMB-I-domain. All the mice receiving PLP-I-domain-1 remained 100% disease-free until the end of the study (Fig. 5C). EAE clinical scores throughout the course of the study did not show any significant difference ($p > 0.05$) between PLP-I-domain-1-treated and Ac-PLP-cIBR7-NH_2-treated mice (Fig. 5A). The clinical scores were significantly lower ($p < 0.05$) in PLP-I-domain-1-treated mice compared to Ac-PLP-LABL-NH_2-treated mice, in spite of the higher dose of Ac-PLP-LABL-NH_2.

It is interesting to find that PLP-I-domain-2 significantly delayed the onset of EAE compared to the PBS-treated group by about 2 days ($p < 0.001$; from day 10 to 16), but was not as potent as PLP-I-domain-1 (Fig. 5A and C). Eventually, there was no significant difference in the disease severity of mice treated with PLP-I-domain-2 and PBS beyond day 16 until the end of the study. Weight loss throughout the course of the study did not show any significant difference ($p > 0.05$) between PLP-I-domain-2-treated mice and those receiving PBS (Fig. 5B). The EAE clinical scores of mice treated with PLP-I-domain-2 were significantly higher ($p < 0.001$) than those treated with Ac-PLP-cIBR7-NH_2 and Ac-PLP-LABL-NH_2. A similar trend was observed in the loss of body weight. Finally, the difference in the *in vivo* efficacy of PLP-I-domain-1 and -2 may be to the difference in the chemical structures of the two molecules.

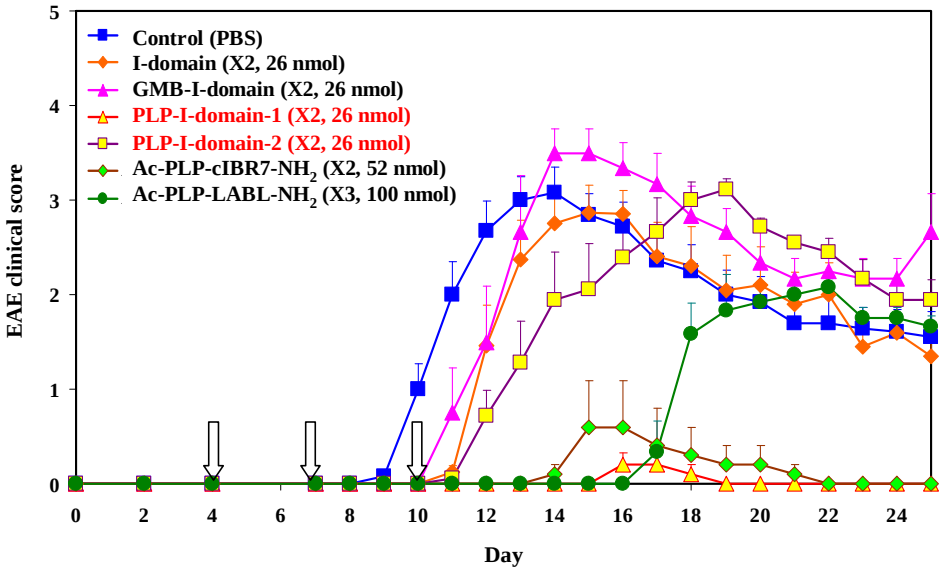

Figure 5A. *In vivo* activity of PLP-I-domain-1 and -2 in mouse EAE model. Disease progression was evaluated using clinical disease scores. After immunization with PLP peptide in CFA, the mice received i.v. injections of 26 nmol of the conjugate PLP-I-domain-1, PLP-I-domain-2, GMB-I-domain, or I-domain on days 4 and 7. For the Ac-PLP-cIBR7-NH2 treatment group, the mice received i.v. injections of 52 nmol of the Ac-PLP-cIBR7-NH2 peptide on days 4 and 7. For the Ac-PLP-LABL-NH2 treatment group, the mice received i.v. injections of 100 nmol of the Ac-PLP-LABL-NH2 peptide on days 4, 7, and 10. The results are expressed as the mean ± S.E. ($n \geq 6$). There are significant differences between PLP-I-domain-1 vs. GMB-I-domain, I-domain, or PBS-treated group in clinical disease score ($p < 0.001$, through days 9–20). There are significant differences between PLP-I-domain-2 vs. PBS-treated group in clinical disease score ($p < 0.001$, through days 9–20). There are significant differences between PLP-I-domain-2 vs. GMB-I-domain or I-domain treated group in clinical disease score ($p < 0.05$, through days 9-20).

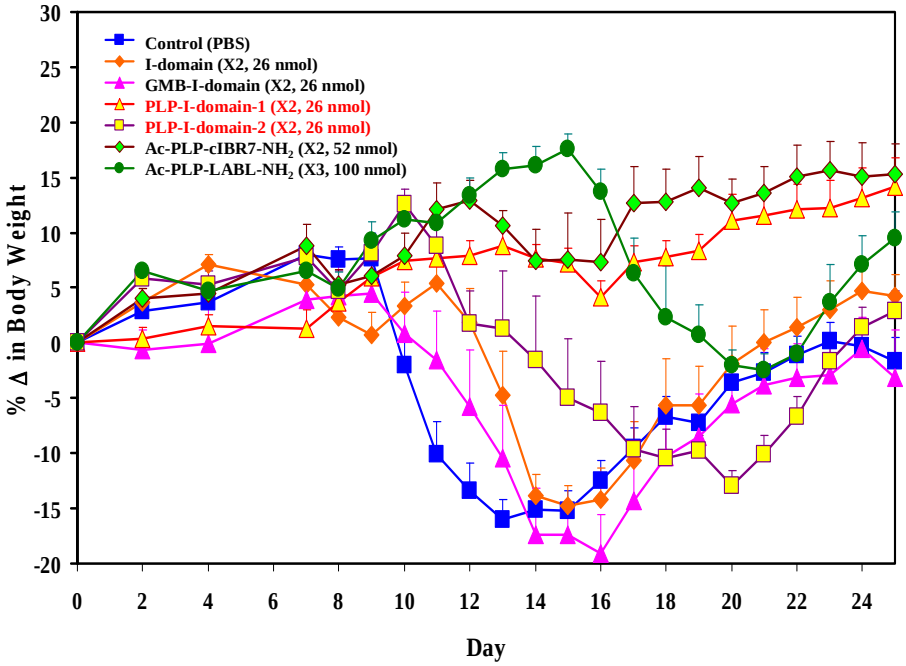

Figure 5B. *In vivo* activity of PLP-I-domain-1 and -2 in mouse EAE model. Disease progression was evaluated using change in body weight. After immunization with PLP peptide in CFA, the mice received i.v. injections of 26 nmol of the conjugate PLP-I-domain-1, PLP-I-domain-2, GMB-I-domain, or I-domain on days 4 and 7. For the Ac-PLP-cIBR7-NH2 treatment group, the mice received i.v. injections of 52 nmol of the Ac-PLP-cIBR7-NH2 peptide on days 4 and 7. For the Ac-PLP-LABL-NH2 treatment group, the mice received i.v. injections of 100 nmol of the Ac-PLP-LABL-NH2 peptide on days 4, 7, and 10. The results are expressed as the mean ± S.E. ($n \geq 6$). There are significant differences between PLP-I-domain-1 vs. GMB-I-domain, I-domain, or PBS-treated group in body weight ($p < 0.001$, through days 9–20). There are significant differences between PLP-I-domain-2 vs. GMB-I-domain or I-domain treated group in body weight ($p < 0.001$, through days 9–20).

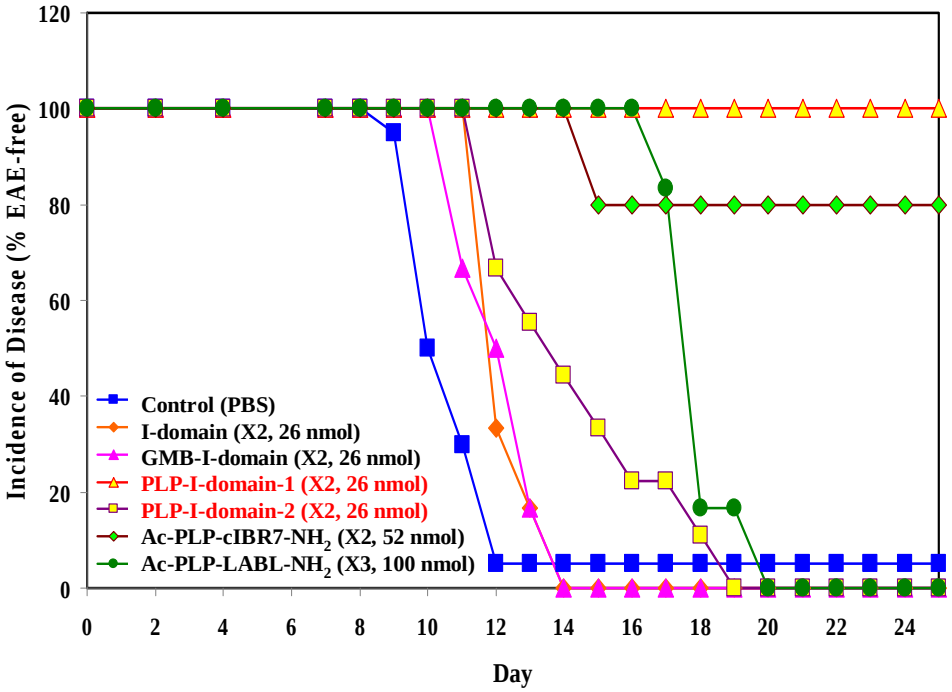

Figure 5C. *In vivo* activity of PLP-I-domain-1 and -2 in mouse EAE model. Disease progression was evaluated using incidence of disease. After immunization with PLP peptide in CFA, the mice received i.v. injections of 26 nmol of the conjugate PLP-I-domain-1, PLP-I-domain-2, GMB-I-domain, or I-domain on days 4 and 7. For the Ac-PLP-cIBR7-NH2 treatment group, the mice received i.v. injections of 52 nmol of the Ac-PLP-cIBR7-NH2 peptide on days 4 and 7. For the Ac-PLP-LABL-NH2 treatment group, the mice received i.v. injections of 100 nmol of the Ac-PLP-LABL-NH2 peptide on days 4, 7, and 10. The results are expressed as the mean ± S.E. ($n \geq 6$). There are significant differences between PLP-I-domain-1 vs. GMB-I-domain, I-domain, or PBS-treated group in disease incidence ($p < 0.001$, through days 9-20). There are significant differences between PLP-I-domain-2 vs. PBS-treated group in disease incidence ($p < 0.001$, through days 9–20).

4.3.4 Structural analysis of PLP-I-domain-1 and -2:

To investigate the structural differences between PLP-I-domain-1 and -2, peptide mapping using tryptic digestion and mass spectrometry was used to determine the location of PLP peptides on the I-domain protein. The assumptions are that the modified lysine residues on the I-domain cannot be cleaved by trypsin, and the cleavaged product of the lysine residue that is attached to the PLP peptide can be used to identify the modified lysine residue on the I-domain. The modified peptide fragments were identified using LC-MS/MS. The PLP peptide contains a Lys residue (Lys12), which also could be hydrolyzed by trypsin to produce a dipeptide, Phe-Cys-OH or Phe-Cys-NH_2, which is attached to maleimidobutyryloxy. These attached dipeptides have molecular weights of 433.1307 Da for Phe-Cys-OH and 432.1467 Da for Phe-Cys-NH_2. In addition, we observed Phe-Cys-OH and Phe-Cys-NH_2 conjugated to the peptide fragment via the hydrolysis product of maleimide which has 18.0106 Da molecular weight added. The comparison of LC-MS/MS data from tryptic-digest fragments of the conjugates is summarized in Table 2. The LC-MS/MS sequence coverage for PLP-I-domain-1 and -2 was 99% and 94%, respectively. A total of 15 modified tryptic peptides were identified from PLP-I-domain-1 and 10 peptides from PLP-I-domain-2. All of the conjugation sites were partially modified, and the number of unmodified peptides dominated the search profile. These modified peptides were unique and were found in the mapping profiles of the conjugates but not in the parent I-domain profile. The experimental and the calculated mass values of the modified peptides were very close, with the average deviation being less than 0.1 Da. There was only one modified peptide missing in the PLP-I-domain-1

Table 2. Modification sites in PLP-I-domain-1 and -2 detected in peptides from trypsin digestion

Modified peptide	Sequence	PLP-I-domain-1	PLP-I-domain-2	Modified sites
T1	^{1}M^{23}K	Yes	Yes	M1
T2-3	^{24}ILDFMKDVM^{33}K	Yes	**No**	K29
T4-5	^{34}KLSNTSYQFAAVQFSTSY^{52}K	Yes	Yes	K34
T6-7	^{53}TEFDFSDYVK^{63}R	Yes	Yes	K62
T8-9	^{64}KDPDALL^{71}K	Yes	**No**	K64
T9-10	^{65}DPDALLKHV^{74}K	Yes	Yes	K71
T12-13	^{96}EELGARPDATKVLIIITDGEATDSGNIDAA^{126}K	Yes	**No**	K106
T13-14	^{107}VLIIITDGEATDSGNIDAAKDII^{130}R	Yes	Yes	K126
T15-16	^{131}YIIGIGKHFQT^{142}K	Yes	Yes	K137
T16-17	^{138}HFQTKESQETLH^{150}K	Yes	**No**	K142
T18	^{151}FASKPASEFV^{161}K	Yes	**No**	K154
T18-19	^{151}FASKPASEFVKILDTFE^{168}K	Yes	Yes	K161
T19-20	^{162}ILDTFEKL^{170}K	Yes	Yes	K168
T20-21	^{169}LKDLFTELQ^{178}K	Yes	Yes	K170
T21-22	^{171}DLFTELQK^{179}K	Yes	**No**	K178
T20-23	^{169}LKDLFTELQKKI^{181}Y	**No**	Yes	K179

that was identified in PLP-I-domain-2. In contrast, there were 6 modified peptides missing in PLP-I-domain-2 that were present in PLP-I-domain-1 (Table 2); for example, Lys154 (K154) was modified in PLP-I-domain-1 but not in PLP-I-domain-2.

4.4 DISCUSSION

Patients with autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and psoriasis are currently being treated with protein-derived drugs such as monoclonal antibodies and peptide polymers, which modulate the immune system. Current treatments of MS patients include Copaxone® and Tysabri® as well as anti-inflammatory agents (*e.g.*, corticosteroids, beta-interferon-1a, mitoxantrone). Some of the drugs for MS have been shown to have different side effects in patients, including the suppression of the general immune response, which can lead to undesirable pathogenic infections. Tysabri® is a monoclonal antibody that binds to the α_4-subunit of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins to block leukocyte adhesion and infiltration into CNS. Although this drug is effective, patients treated with Tysabri® were found to develop progressive multifocal leuko-encephalopathy (PML), a life-threatening complication in patients.[14] PML was also observed in patients treated with Raptiva® (Efalizumab, CD11a mAb) for psoriasis; thus, this drug was withdrawn from the market.[15-16] Because Tysabri® and Raptiva® bind to integrins, these antibodies presumably also suppress Signal-2 for T-cell activation in addition to blocking the cell adhesion. Blocking Signal-2 of the immunological synapse formation suppresses the general activation of T cells that can respond to pathogens such as JC virus that causes PML. Therefore, there is a need to discover a new way to suppress

T-cell activation in an antigen-specific manner without suppressing the general immune responses.

To address the issue of antigen specific disease suppression while preserving the immune system's ability to fight to foreign pathogens, our group developed BPI molecules (GAD-BPI, PLP-BPI, and CII-BPI),[3-7] which were derived from antigenic peptides discovered by others.[17-22] In parallel with the design of BPI molecules, the PLP-I-domain molecules were developed by attaching several antigenic peptides to one molecule of I-domain. Thus, the advantage of making PLP-I-domain is that the I-domain can be used to carry multiple copies of the antigenic peptides to improve the potency of the conjugate. In addition, the I-domain can also be utilized to carry multiple and different antigenic peptides to modulate different subpopulations of antigen-specific T cells. Similar to BPI, PLP-I-domain conjugates are hypothesized to inhibit the immunological synapse formation during the process of T-cell activation by simultaneous binding of the PLP peptide and I-domain to MHC-II and ICAM-1, respectively, on APC. This simultaneous binding forms a bridge between the two receptors and eventually prevents the translocation and reorganization of Signal-1 and Signal-2.

In the present study, it was found that just two i.v. injections (26 nmol/injection) of PLP-I-domain-1 inhibited the onset and progress of EAE more efficiently than PBS alone or three injections (100 nmol/injection) of Ac-PLP-LABL-NH_2. Also, there was no significant difference between two injections of PLP-I-domain-1 and Ac-PLP-cIBR7-NH_2. The improved efficacy of PLP-I-domain-1 could be due to the higher binding efficiency of I-domain to ICAM-1 than that of LABL peptide in Ac-PLP-LABL-NH_2 or

it could be due to the delivery of multi-antigenic peptides. Thus, the mechanisms of the way that PLP-I-domain-1 regulates the autoimmunity are currently being investigated.

Another conjugate, PLP-I-domain-2, was synthesized using PLP-Cys-NH_2 peptide, which has an amide group on the C-terminus. The optimal conjugation of PLP-Cys-NH_2 to I-domain was at pH 6.6. It is interesting to find that two i.v. injections (26 nmol/injection) of PLP-I-domain-2 produce some delay in the onset and severity of the disease compared to PBS, but not to the same extent as PLP-I-domain-1. PLP-I-domain-2 is also less potent than Ac-PLP-LABL-NH_2 and Ac-PLP-cIBR7-NH_2. The analyses of both PLP-I-domain-1 and -2 indicate that both molecules contain an average of 2.5 peptide molecules per I-domain molecule. The SDS-PAGE and CD analyses could not differentiate between the two molecules. Thus, it is possible that the difference in *in vivo* activity of these two molecules could be due to the locations of the conjugated peptides and/or the nature of the C-terminal form of PLP peptide—whether it is capped with amide or in free carboxylic acid form.

To determine the difference in the conjugation sites, both conjugates and the parent I-domain were subjected to tryptic digestion followed by LC-MS/MS analysis. PLP-I-domain-1 has a total of 15 lysine sites that are modified by PLP-Cys-OH peptide compared to PLP-I-domain-2 with 10 lysine sites that are modified by the PLP-Cys-NH_2. The difference in the pH of reaction (8.5 and 6.6) could contribute to the location of conjugation on the GMB-I-domain protein (Step 2). It has been shown that reactivity of thiol group in attacking the maleimide group is accelerated by increasing the pH.[13] This is consistent with the higher number of conjugation sites on PLP-I-domain-1 compared to

PLP-I-domain-2. At this time, it has not yet been determined whether the nature of the C-terminus of the PLP peptide has an effect on the conjugation sites. It is most likely that the nature of the peptide C-terminus does not contribute greatly to the number of sites of conjugation.

There are sites of peptide conjugation present in PLP-I-domain-1 that are not found in PLP-I-domain-2; these sites include K29, K64, K106, K142, K154, and K178 (Fig. 6). The observable difference in the activity of PLP-I-domain-1 and PLP-I-domain-2 suggests that conjugation of PLP peptides on K29, K64, K106, K142, K154, and K178 contributes to the activity of PLP-I-domain-1, and the absence of peptides on these sites in PLP-I-domain-2 makes it less potent to suppress EAE. The difference in the C-terminus of PLP peptide may not contribute to the lower activity of PLP-I-domain-2; our previous studies showed that amidation of the C-terminus BPI molecules containing PLP peptide enhanced the *in vivo* activity of BPI molecules. The locations of the modified K29, K64, K106, K142, K154, and K178 are indicated in the structure of I-domain (yellow ribbons, Fig. 7). If the activity of PLP-I-domain-1 is due to its simultaneous binding to ICAM-1 and MHC-II on the surface of APC, the most active conjugate should accommodate binding to these two receptors. It is known that the I-domain binds to ICAM-1 via its MIDAS region (magenta, Fig. 7). From the model derived from the X-ray structure, it can be predicted that conjugation of PLP peptide at K142 and K178 would be the most probable sites to accommodate simultaneous binding of PLP-I-domain-1 to MHC-II and ICAM-1. In the future, the most important site(s) for peptide conjugation will be determined using a single mutation of each Lys residue (i.e., K29, K64, K106,

PLP-I-domain-1

```
         10          20          30          40          50          60
MGNVDLVFLF DGSMSLQPDE FQKILDFMKD VMKKLSNTSY QFAAVQFSTS YKTEFDFSDY
         70          80          90         100         110         120
VKRKDPDALL KHVKHMLLLT NTFGAINYVA TEVFREELGA RPDATKVLII ITDGEATDSG
        130         140         150         160         170         180
NIDAAKDIIR YIIGIGKHFQ TKESQETLHK FASKPASEFV KILDTFEKLK DLFTELQKKI

Y
```

PLP-I-domain-2

```
         10          20          30          40          50          60
MGNVDLVFLF DGSMSLQPDE FQKILDFMKD VMKKLSNTSY QFAAVQFSTS YKTEFDFSDY
         70          80          90         100         110         120
VKRKDPDALL KHVKHMLLLT NTFGAINYVA TEVFREELGA RPDATKVLII ITDGEATDSG
        130         140         150         160         170         180
NIDAAKDIIR YIIGIGKHFQ TKESQETLHK FASKPASEFV KILDTFEKLK DLFTELQKKI

Y
```

Figure 6. The amino acid sequence of PLP-I-domain-1 and -2. Conjugation sites detected in trypsin digestion are shown in bold, red, and underlined letters. The missing conjugation sites are shown in bold, blue, and underlined letters.

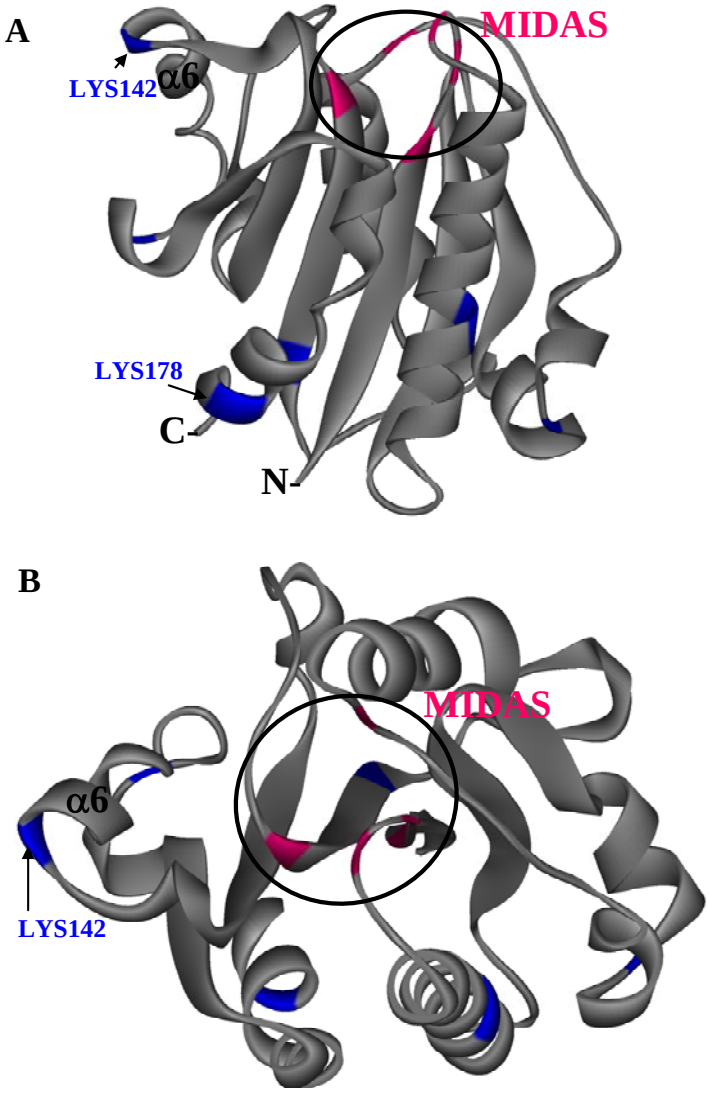

Figure 7. The X-ray structure of I-domain (PDB code 1ZON). The residues in blue are the lysine modification sites found in PLP-I-domain-1 but not in PLP-I-domain-2 and the residues in pink are located in the MIDAS region. The N- and C-termini are labeled as N- and C-, respectively. The protein images were created using Accelrys DS Visualizer 1.7. **(A)** Side view. **(B)** Top view.

K142, K154, or K178) to a Cys residue followed by conjugation of PLP peptide. In this case, the peptide will be derivatized with a maleimide group. To illustrate, Lys142 can mutated with Cys142 to give Cys142-I-domain. Then, Cys142-I-domain will be conjugated with PLP peptide to give PLP-Cys142-I-domain, and the *in vivo* activity of this new conjugate will be determined in the EAE mouse model. Using this method, we can pinpoint the conjugation site(s) that is important for the activity of the PLP-I-domain molecule. It is possible that multiple sites of peptide conjugation are necessary; in such a case, multiple Cys mutations can be carried out on a single I-domain protein.

4.5 CONCLUSIONS

We have shown the proof-of-concept that PLP-I-domain-1 has excellent efficacy in inhibiting the progress of EAE in the mouse model. This study also indicates that the location(s) of peptide conjugation on the I-domain has an impact on the *in vivo* activity. In the future, we will study the mechanism of action of PLP-I-domain-1 in regulating the immune response in EAE. If the PLP-I-domain has a mechanism of action similar to that of BPI molecules, we expect that treatment of EAE mouse with PLP-I-domain-1 will increase the production of IL-10, IL-4, and TGF-β cytokines, which possibly tips the balance from T_H1 and T_H17 to T_{reg} and T_H2 differentiations. Another consideration is that the presence of antigen spreading in autoimmune diseases such as MS can be solved with multiple sites of conjugation with different peptides on I-domain protein. For example, antigenic peptides PLP, myelin basic protein (MBP), and myelin oligodendrocyte

glycoprotein (MOG) can be simultaneously conjugated to I-domain to cover different subpopulations of T cells. In such conditions, conjugation of each I-domain molecule with all the three immunodominant epitopes may offer a unique approach for the treatment of MS.

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CHAPTER 5

Summary, Conclusions, and Future Directions

SUMMARY AND CONCLUSIONS

The objective of the current work was to utilize the I-domain protein to deliver antigenic peptides (i.e., PLP peptide) to control EAE in an antigen-specific manner in the mouse model for MS. I-domain is a well characterized protein; it contains a major ICAM-1 binding site and can block T-cell binding to immobilized recombinant ICAM-1-Fc.[1-3] The I-domain protein has a unique divalent cation coordination site called metal ion-dependent adhesion site (MIDAS) that interacts with the D1 domain of ICAM-1.[1, 4-6] Up to this point, the binding and internalization properties of the soluble I-domain to ICAM-1 on the cell surface have not been studied. Furthermore, its utility to target drugs to lymphocytes has not been explored.

In this work, we conjugated a fluorescein molecule (FITC) to I-domain protein for studying its ICAM-1-binding and uptake properties on B-lymphocytes (Raji cells). The data were used to predict I-domain applicability for targeting drug molecules to cells with upregulated ICAM-1 expression. The FITC conjugation was to the ε-amino group of the lysine residues and the N-terminus of the I-domain to generate FITC-I-domain. The binding of FITC-I-domain to ICAM-1 on Raji cells was blocked by anti-I-domain mAb, suggesting that the antibody binds to the ICAM-1 binding site of the I-domain. In contrast, incubation of an anti-ICAM-1 mAb on the cells enhanced the binding of FITC-I-domain to ICAM-1; this result suggests that anti-ICAM-1 mAb has an allosteric effect on I-domain binding. A detailed structural analysis of FITC-I-domain using tryptic digestion followed by LC MS analysis showed that the FITC modifications were found at the lysine residues away from the MIDAS region. These findings indicate that FITC

derivatization of the I-domain does not influence the binding properties of FITC-I-domain to ICAM-1 on Raji cells.

To determine the cellular uptake of FITC-I-domain, confocal microscopy studies followed by cell image analyses were performed using Raji cells incubated with FITC-I-domain at either 4 or 37 °C with increasing incubation time. This study showed that the average integrated fluorescence intensity values associated with the cytoplasm were increasing with the incubation time at 37 °C but not at 4 °C. These results indicate that FITC-I-domain internalizes in Raji cells via an energy-dependent process.

The applicability of the I-domain conjugate to deliver drug molecules or peptides to antigen-presenting cells (APC) was evaluated in the EAE animal model, a model for multiple sclerosis. In this case, antigenic peptides from proteolipid protein (PLP) were conjugated with the I-domain to produce PLP-I-domain-1 and -2. PLP-I-domain-1 and -2 were prepared by conjugating PLP-Cys-OH and PLP-Cys-NH_2 at pH 8.5 and 6.6, respectively, to the I-domain. Both the conjugates had similar characteristics as determined by SDS-PAGE, mass spectrometry, and CD analysis. In mice treated with two i.v. injections (26 nmol on days 4 and 7) of PLP-I-domain-1, the onset and progression of EAE were inhibited more significantly than in PBS-treated mice. Mice treated with PLP-I-domain-2 showed some delay in the onset and severity of EAE compared to PBS, but PLP-I-domain-2 has a lower efficacy than PLP-I-domain-1. PLP-I-domain-1 but not PLP-I-domain-2 was found to have better potency than three i.v. injections of 100 nmol of Ac-PLP-LABL-NH_2 administered on days 4, 7, and 10. PLP-I-

domain-1 activity was similar to that of Ac-PLP-cIBR1-NH_2 (i.e., 52 nmol i.v. injections on days 4 and 7).

To investigate the structural differences between the two conjugates (PLP-I-domain-1 and -2), they were subjected to tryptic digestion and LC-MS/MS analysis. The results show that PLP-I-domain-1 has a total of 15 modification sites in contrast to PLP-I-domain-2, which has 10 modification sites. The difference in the number of modification sites could be due to differences in their reaction conditions (i.e., pH 8.5 and 6.6) and, to a lesser extent, to the difference in the C-termini of the PLP peptides. The additional conjugation sites on PLP-I-domain-1 compared to PLP-I-domain-2 may contribute to the excellent *in vi*vo activity of PLP-I-domain-1.

In conclusion, the current work is the first study to show that I-domain can bind and enter into the cytoplasmic compartment of immune cells; therefore, it can be utilized to deliver drugs to ICAM-1-expressing target cells. Most importantly, PLP-I-domain-1 can suppress the progression of EAE in the mouse model. The fact that PLP-I-domain-1 has superior activity compared to PLP-I-domain-2 suggests that the location(s) of peptide conjugation on the I-domain protein have a significant impact on the conjugate efficacy.

FUTURE DIRECTIONS

Exploring the possible mechanisms of action of PLP-I-domain conjugate:

In vivo studies in EAE mice indicate that treatment of mice with Ac-PLP-LABL-NH_2 significantly increases the TGF-β, IL-10, IL-4 production and decreases the IFN-γ production compared to treatment with PBS.[7] We predict that treatment with PLP-I-

domain suppresses the IFN-γ producing T_H1 cells and induces the differentiation of TGF-β- and IL-10-producing T_{reg} cells and IL-4 producing T_H2 cells. We propose that there is a shift in the balance from immunogenic to immnuotolerant response. Because of the elevated levels of IL-17 in patients with MS, it would be interesting to determine the plasma levels of IL-17 and the presence of T_H17 cells in the EAE animal model upon treatment with PLP-I-domain-1. In the future, we will determine the plasma cytokine levels (IL-10, TGF-β, IL-4, IFN-γ, and IL-17) along with the presence of different T-cell subpopulations (T_H1 and T_H17 vs. T_H2 and T_{reg}) in mice treated with PLP-I-domain-1 and PBS. The results from these proposed studies will shed light on the potential mechanism(s) of action of PLP-I-domain-1.

Conjugation of antigenic peptide at a specific site on I-domain:

It is important to determine the crucial modification site(s) on the I-domain protein that contributes to the activity of PLP-I-domain-1. Our hypothesis is that the activity of PLP-I-domain may be due to its simultaneous binding of PLP-I-domain-1 to MHC-II and ICAM-1 on the surface of APC. Chapter 4 provides insight into probable site(s) where PLP conjugation may facilitate such simultaneous binding. For example, attachment of PLP peptide on Lys142 was found on PLP-I-domain-1 but not on PLP-I-domain-2. It should be noted that Lys14 is adjacent to the MIDAS region on I-domain. Thus, the conjugation of PLP peptide at Lys142 may accommodate binding of PLP-I-domain conjugate to MHC-II and ICAM-1 on APC to inhibit the immunological synapse formation. To test this hypothesis, we will selectively mutate Lys142 to Cys142 to give

Cys142-I-domain. Then, the thiol group of Cys142 in Cys142-I-domain will be conjugated to PLP peptide containing a maleimido group to produce PLP-Cys142-I-domain conjugate (Fig. 1). It is expected that the PLP-Cys142-I-domain will be a homogeneous product because the original I-domain protein has no cysteine residue. The *in vivo* efficacy of PLP-Cys142-I-domain will be compared to that of PLP-I-domain-1 and PBS in the mouse EAE model. Other single mutations and conjugations will be evaluated *in vivo*. These studies will locate the important peptide conjugation site(s) on the I-domain protein that contribute to the *in vivo* activity of the conjugate. Furthermore, multiple mutations can be carried out in I-domain for delivering multiple antigenic peptides to APC to overcome the potential antigen spreading in multiple sclerosis (see below).

Application of antigenic peptide-I-domain conjugates for suppression of other autoimmune diseases:

The general applicability of antigenic peptide-I-domain to treat different autoimmune diseases such as rheumatoid arthritis (RA) and type 1 diabetes (T1D) may be tested by conjugating the I-domain to an antigenic peptide specific for that particular disease. For example, the antigenic peptide (i.e., PLP-) in PLP-I-domain can be replaced with antigenic peptide from collagen-II ($CII_{256–270}$) antigen and with glutamic acid decarboxylase ($GAD_{208–217}$) antigen to prepare CII-I-domain and GAD-I-domain, respectively. The efficacies of CII-I-domain and GAD-I-domain can be evaluated respectively in the collagen-induced arthritis (CIA) mouse (a model for RA) and non-

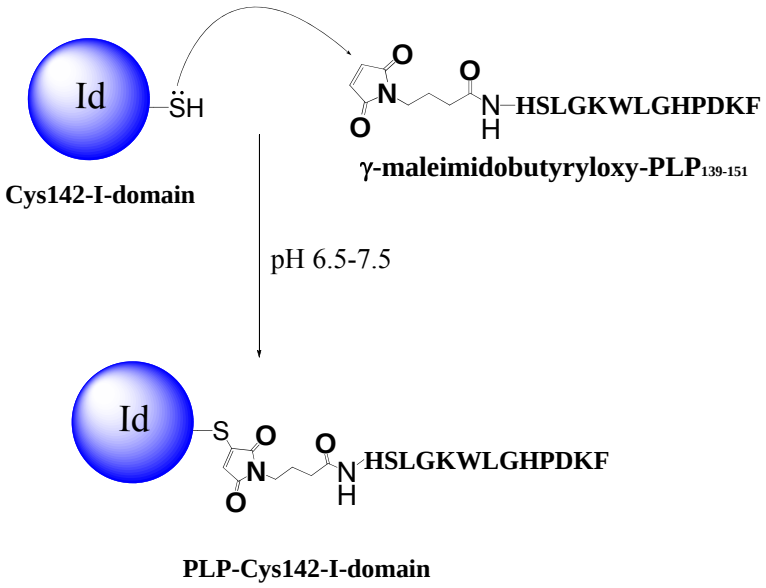

Figure 1. Schematic representation of a single-step modification of Cys142-I-domain mutant with γ-maleimidobutyryloxy-PLP139-151 to produce PLP-Cys142-I-domain conjugate.

obese diabetes (NOD) mouse (a model for T1D). It will be interesting to evaluate whether there is a common general mechanism of action of antigen-I-domain conjugates in different autoimmune diseases.

Targeting multivalent antigenic peptides using I-domain protein:

One of the main reasons why autoimmune diseases are considered to be complex is because of the possibility of antigen or epitope spreading. In such cases, immune cells change their response from targeting the primary epitope to targeting many different epitopes on an autoantigen.[8-10] Epitope spreading may complicate the application of specific therapies designed to interfere with immune cells that recognize a single antigen. One possible solution to this scenario is to develop a novel therapy that can cover different subpopulations of T cells that recognize different self antigens. In this case, the I-domain protein is an excellent choice for conjugating multiple antigenic peptides on the lysine residues. For example, antigenic peptides from PLP, myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG) are simultaneously conjugated to a single I-domain molecule (Fig. 2) to give a mixture of PLP/MBP/MOG-I-domain that can respond to suppress the proliferation of subpopulations of inflammatory T cells, which are sensitive to these various antigens in the EAE mouse model.

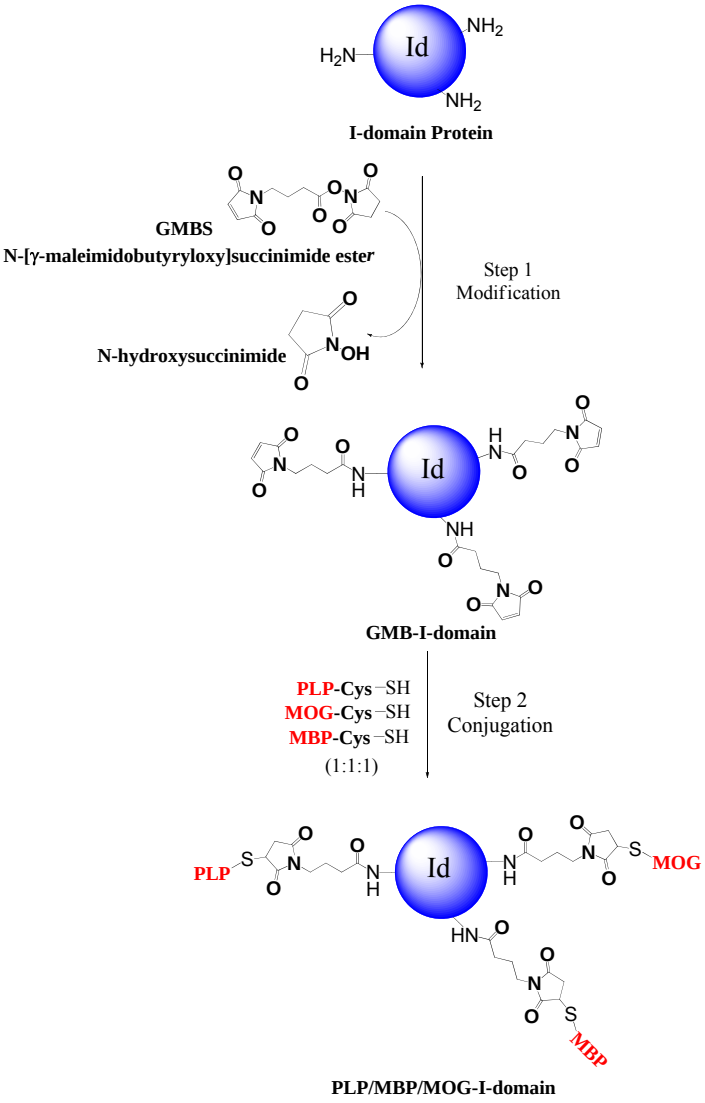

Figure 2. Schematic representation of modification and conjugation reactions in the preparation of a multivalent antigenic peptide-I-domain conjugate. **Step 1:** Modification of the I-domain protein with GMBS. **Step 2:** Conjugation of PLP, MBP, and MOG to GMB-I-domain.

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